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PRINCIPAL INVESTIGATOR: Insoo Bae, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057-1411

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Insoo Bae, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057-1411

E-Mail: ib42@georgetown.edu

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13. ABSTRACT (Maximum 200 Words)

Gadd45, a DNA damage inducible gene, plays role(s) in DNA repair and regulation of cell proliferation. Using yeast two hybrid system, we identified a novel gene whose products interact with GADD45α and named it as GIF1 (Gadd45 Interacting Factor 1). Alternative name of GIF1 is CRIF1 (=CR6 interacting factor 1, CR6=Gadd45γ). During the first year of funding period, we reported that a physical interaction of Gadd45 family proteins and GIF1 in mammalian cells using IP and GST pulldown assay. In the same paper, we also reported that GIF1inhibits the cyclin-dependent kinases, Cdc2/cyclin B1 kinase and Cdk2/cyclin E, so it renders inhibition of the cell cycle progression at the G1/S phase and suppresses growth in serum stimulated NIH3T3 cells. In order to introduce GIF1 efficiently into breast cancer cells, we constructed rAd-GIF and confirmed that exogenous GIF expression in breast cancer cell lines. Infection of rAd-GIF1 increased cells in G1 phase in human cells. To extend our study we performed another round of yeast hybrid screening and found GIF interact with two other nuclear hormone receptor, NUR77 and Androgen receptor. We submitted a manuscript titled as "CR6 Interacting Factor 1 (CRIF1) inhibits with Orphan Nuclear Receptor Nur77 and inhibits its transactivation". Before initiating Task3 (role of GIF1 in response to cytotoxic agents), we examined whether GIF1 mRNA is inducible in response to stress such as X-ray, MMS, UVB, H_2O_2, ADR (adriamycin), Thapsigargin, Taxol, Camptothecin, Cis-Platinum, TPA, Nacl, Heat shock, Arsenic in TK6 (p53wt), NH32 (p53 KO) and MCF-7 breast cancer cells. In this experiments we concluded that GIF1 is not a stress inducible gene.

14. SUBJECT TERMS
GIF1, CRIF1, Gadd45, adenovirus, siRNA, NUR77

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ARMY MEIDCAL RESEARCH AND MATERIAL COMMAND: ANNUAL REPORT (SECOND YEAR's REPORT)

PI: Insoo Bae
Title: Role(s) of GIF1 (GADD45 Interacting factor 1) in Breast Cancer

INTRODUCTION:

Using yeast two hybrid system, we identified a novel gene whose products interact with GADD45α and named it as GIF1 (Gadd45 Interacting Factor 1) (1). Alternative name of GIF1 is CRIF1 (=CR6 interacting factor 1, CR6=Gadd45γ). It has been identified as a protein that interacts with the papilloma virus type 16 L2 protein (2). Although GIF1exhibits the ubiquitous expressions, high level of GIF1 expression is observed in the endocrine organs, adrenal glands, thyroid, testes and ovaries (1 or AM-1). The studies on intracellular localizations of GIF1 revealed that it is mainly expressed in the nucleus (1,2). However, the intranuclear actions of GIF1 have not been fully elucidated. For the first year of funding, we reported that GIF1 inhibits the cyclin-dependent kinases, Cdc2/cyclin B1 kinase and Cdk2/cyclin E, so it renders inhibition of the cell cycle progression at the G1/S phase and suppresses growth in serum stimulated NIH3T3 cells (1). In order to introduce GIF1 efficiently into breast cancer cells, we constructed rAd-GIF. Infection of rAd-GIF1 increased cells in G1 phase in human cells. Using siRNA for GIF1, we successfully knockdown endogenous GIF1 in MCF-10A human breast cancer cells. We examined whether GIF1 mRNA is inducible in response to stresses such as X-ray, MMS, UVB, H_2O_2, ADR (adriamycin), Thapsigargin, Taxol, Camptothecin, Cis-Platinum, TPA, Nacl, Heat shock, Arsenic in TK6 (p53wt), NH32 (p53 KO) and MCF-7 breast cancer cells. GIF1 mRNA was not stress-inducible gene.

To extend our study we performed another round of yeast hybrid screening and found GIF interact with two other nuclear hormone receptor, NUR77 and Androgen receptor. But GIF1 did not binds to GIF1 itself, indicating that it does not form heterodimers. We submitted a manuscript titled as "CR6 Interacting Factor 1 (CRIF1) inhibits with Orphan Nuclear Receptor Nur77 and inhibits its transactivation" (AM-2).

BODY:

APPROVED STATEMENT OF WORK

Task 1. To determine the association between GADD45 and GIF1 (Month 1-24).
- Screen breast cancer cell lines expressing low basal level of GIF1 by RT-PCR and Western blotting. Month 1-6.
- Construct stable cell lines overexpressing GIF1. Month 6-12.
- Determine GIF1 and Gadd45 interaction using Immunoprecipitation. Month 12-18.
-Construct GIF1 and GADD45 deleted and point mutants for mapping. Month 18-24.

Task 2. To examine and compare the effects of GIF1 cDNA transgenes on breast cancer cell lines (Months 12-22).
- Determine Effects on proliferation. Month 12-18.
-Determine Effects on cell cycle kinetics. Month 18-22.
-

Task 3. To study of role of GIF1 in the human breast cancer cell response to cytotoxic agents. (Months 18-36).
-Determine Effects on cell viability. Months 18-30.
-Determine Effects on apoptosis. Month 24-30.

* Organizing data and prepare a final report (Months 30-36).

Appended Manuscripts and Publications

AM-1. Chung HK, Yi YW, Jung NC, Kim D, Suh JM, Kim H, Park KC, Song JH, Kim DW, Hwang ES, Yoon SH, Bae YS, Kim JM, Bae I, Shong M. CR6 Interacting Factor 1 (CRIF1) interacts with Gadd45 family proteins and modulates the cell cycle. 2003. J Biol Chem. 2003 Jul 25;278(30):28079-88. Epub 2003 Apr 25.

In this publication we have demonstrated that GIF1 (=CRIF1) can physically interact with three Gadd45 family proteins, Gadd45α, Gadd45β and Gadd45γ in vitro and in vivo. We also found that GIF1 can arrest cell in G1 phase possibly by activating RB (dephosphorylation) and inhibit cell proliferation. These findings are described in detail in Appended Manuscript 1 (AM-1, Chung et al.) and summarized below. We have essentially completed the major goals with Tasks 1 and 2, as outlined below. However, most of data presented in AM-1 utilized non-breast cancer cells due to a difficulty of transfection of GIF1 in breast cancer cells. To overcome the transfection problem, we developed several reagents that can be used for GIF transfection and Kockdown approach.

AM-2 Park KC, Song KH, Chung HK, Kim H, Kim DW, Song JH, Hwnag ES, Chung H, Park SH, Bae I, Choi HS, Shong M. CR6 interacting Factor 1 (CRIF1) interact with Orphan Nuclear Receptor Nur77 and inhibits its transactivation. Accepted in Mol. Endocrinology.

In this study we made a novel function of GIF1. In this study we demonstared that GIF1 acts as a novel co-regulator of orphan nuclear receptor Nur77 transactivation. Both in vitro and in vivo studies show that GIF-1 interacts with Nur77. The GIF1 interacts with distinct AB domain of Nur77 and it dramatically inhibited the AB domain-mediated transactivation of Nur77. Transient transfection assay demonstrate that GIF1 inhibit Nur77 transactivation and

repress SRC-2-mediated Nur77 transactivation. The silencing of the endogenous CRIF1 by siRNA resulted in the activation of Nur77-mediated transactivation. The GIF1 posses intrinsic repressor activities, but the repressive activities on

Summary of Major Findings During the Second Year of Grant Period (Task Address):

Studies addressing Task1.

1. We planed to construct stable cell lines overexpressing GIF1 using breast cancer cell lines. However, we were not successful in making a stable cell line expressing GIF1 in breast cancer cell line. This might be, in part, due to a growth suppressive property of GIF1 (1). Thus, we developed recombinant adenovirus expressing GIF1 at high levels in human tumor cell lines; MDA-MB-231 (breast), MCF-10A (breast), A549 (lung), HS27 (skin fibroblast) and HS68 (skin fibroblast) (Fig. 1).
2. We confirmed physical interaction of GIF and Gadd45 protein using IP and GST pulldown assay (1). In order to map precise binding sites of Gadd45 on GIF1 and visa versa, we constructed a series of GIF1 deletion mutants (Fig. 2).

Studies addressing Task 2. Main goal of task2 was to determine effects of GIF on cell proliferation and cell cycle kinetics. To accomplish task2 we performed the following experiments.

1. We finished screening of breast cancer cell lines by RT-PCR and Western blotting. We found that breast cancer cells express GIF1 mRNA and protein. MCF7 is cell line that expresses the least GIF1. The level of GIF1 protein expression was slightly higher in prostate cancer cell lines (LnCaP, DU-145 and PC-3) and one Kidney cell line (293) cells comparing to breast cancer cell lines (Fig. 3).
2. Gadd45 proteins bind Cdc2 kinase which inhibits cellular proliferation (3). Since GIF1 and Gadd45 proteins interact in our study, Cdc2 kinase activity was examined in the presence or absence of Gadd45 proteins and GIF1. As shown in Figure 4 (AM-1), recombinant Gadd45α and Gadd45γ rescued histone H1 kinase activity in Cdc2/Cyclin B1 immunoprecipitates to approximately 60% of the control (the presence of Cdc2/Cyclin B1 was demonstrated by immunoblot; data not shown). Addition of recombinant GIF1 resulted in a progressive inhibition of the enzymatic activity of the immunoprecipitated Cdc2/Cyclin B1 complex. Unlike Gadd45, GIF1 inhibit Cdk2/Cyclin E histone H1 kinase activity. Gadd45 proteins have no effect on GIF-1 mediated inhibition of Cdk2/Cyclin E histone kinase activity (Fig 4B in AM-1).
3. Since GIF1 can inhibit the activity of Cdc2/Cyclin B1 and Cdk2/Cyclin E complexes, it is possible that GIF1 can also influence cell cycle progression. This idea was tested by overexpressing GIF1 in NIH3T3 cells and monitoring cell cycle progression by flow cytometry. LipofectAmince plus was used to reproducibly transfect NIH3T3 cells with pFlag-GIF1 with an efficiency of 30 to 50% (Fig. 5A in AM-1). Cell cycle progression was analyzed within 1-2 days after transfection. pFlag-GIF1 and pCMV-Tag2 (vector control) were trasfected into NIH3T3 cells and GIF1 expression was measured by immunoblotting with mouse anti-Flag antibody. As results, we found that cells overexpressing GIF1 (transfected with 4μg of pFlag-GIF1) were more likely to be in G1 and less likely to be in S phase than control cells (Fig. 5A in AM-1). In order to confirm our finding in human cell lines we constructed recombinant adenovirus expressing GIF1. As shown in Figure 2A,

infection of rAd-GIF1 express high levels of GIF1. First we used two human skin fibroblast to investigate whether overexpression of GIF1 cause growth arrest in human cell lines. We found that overexpression of rAd-GIF1 arrest cells in G1 (Table 1). Currently we are testing human breast cell lines confirm role of GIF1 in cell cycle progression in human breast cancer cells.

4. The growth rate was also monitored in cells overexpressing pFlag-GIF1. NIH3T3 cells were cotranfected with pCMV-Tag2 or pFlag-GIF1 and pCMV-puro and grown for 5 days; pCMV-puro encodes a gene that confers puromycin resistance for selection purposes during cell growth. Cells transfected with pFlag-GIF1 grew more slowly than control cells transfected with pCMV-Tag2 (Fig 5B in AM-1). After 5 days of selection, the number of cells overexpressing pFlag-GIF1 was about half of control cells.
5. We also studied the role of endogenous GIF1 in the regulation of cell proliferation and cell cycle by using 21-nucleotide duplexes of siRNA. We selected four different target regions of human GIF1 for the synthesis of RNA duplexes (Fig. 6A in AM-1). As results, we found that siRNA-263 and –379 are effective siRNA duplexes for the suppression of exogenous and endogenous GIF1 expression.
6. We observed the role of GIF1 in the phosphorylation of Rb by overexpression and downregulation of GIF1. As shown in Fig 7A in AM-1, GIF1 expression resulted decrease of phsphorylated forms of RB with the increase the levels of GIF1 expression. The decrease of phosphorylated RB by GIF1 overexpression was completely inhibited by GIF1-siRNA-263 and-379 cotransfection, but not by GIF1-siRNA-578 which was not able to suppress the GIF1 expression (Fig. 8B in AM-1). These siRNA experiments suggest that modulation of intracellular level of GIF1 results in the regulation of G1/S phase of cell cycle.
7. Since it was technically difficult to transfect and express GIF cDNA in human cells, we will use siRNA for GIF1. We transfect siRNA for GIF into MCF-10A and found that about 50% of GIF1 are knockdown (Fig. 4). We will continue our study with siRNA to determine role(s) of GIF1 in breast cancer cell proliferation. In addtion to that, we construct GIF1 siRNA expressing recombinant retrovirus and we are trying to make GIF KO breast cell line (data not shown).

Task 3. Studies addressing Task3. A major goal for Task 3 is to study of role of GIF1 in the human breast cancer cell response to cytotoxic agents. Before investigating function of GIF1 in response to cytotoxic agents, we examined whether GIF1 mRNA is inducible in response to stress such as X-ray, MMS, UVB, H2O2, ADR (adriamycin), Thapsigargin, Taxol, Camptothecin, Cis-Platinum, TPA, Nacl, Heat shock, Arsenic in mammalian cells. Not significance chance in expression of GIF was obvious in response to various drug treatement and environmental stresses. In this experiments we concluded that GIF1 is not a stress inducible gene (Table 2).

Studies outside of Task.

Interaction with other nuclear hormone receptor, AR and NUR77 (Yeast 2-hybrid system).
Previously we reported that Gadd45 family proteins interact with a various nuclear hormone receptor (4). Since GIF1 interact with Gadd45 family protein (1), we investigated physical interaction of GIF1 with various nuclear hormone receptors. The plasmids encoding the Lex A-DBD fusion of GIF1, ERRγ, GR, SHP, mAR and SF-1 with the plasmids encoding the B42-AD fusion of CRIF1, RXR, CAR, TR, SMRT and Nur77 were transformed into the yeast strain, EGY48. As shown in Table 1 (in AM-2), the LexA fusion proteins including GIF1 strongly interact with B42 activation domain fused to Nur77. The mouse AR (androgen receptor) also has interactions with GIF1 in the presence or absence of a ligand.
Effect of GIF1 on regulation of NUR77-mediated gene expression.
In this study we made a novel function of GIF1. In this study we demonstared that GIF1 acts as a novel co-regulator of orphan nuclear receptor Nur77 transactivation. Both in vitro and in vivo studies show that GIF-1 interacts with Nur77. The GIF1 interacts with distinct AB domain of Nur77 and it dramatically inhibited the AB domain-mediated transactivation of Nur77 These results suggest that GIF might act as a repressor of the orphan nuclear receptor Nur77 by inhibiting the AB domain-mediated transcriptional activities (AM-2).
No effect on ER signaling pathway. Potential roles of Gadd45 family genes and GIF1 in ER signaling pathway have been investigated by a promoter study. As results, we found that Gadd45 family proteins have inhibitory effects on ER signaling pathway. However, GIF1 does not affect on ER signaling pathway. (Data not shown).
No effect on NFKB singling pathway. Potential roles of Gadd45 family genes and GIF1 in NFKB signaling pathway has been investigated and found that GIF does not affect on NFKB signaling pathway. (Data not shown).

Key Research Accomplishments

- We successfully constructed rAd-GIF and confirmed that exogenous GIF expression in breast cancer cell lines (Fig. 1). We confirmed that overexpression of GIF arrested cells in G1 phase in human cell lines such as HS27 and HS68 human skin fibroblast cells (Table 1).
- We completed construction of GIF1 deletion mutants that will be used to map Gadd45 binding site on GIF1 (Fig. 2)
- We examined whether GIF1 mRNA is inducible in response to stress such as X-ray, MMS, UVB, H2O2, ADR (adriamycin), Thapsigargin, Taxol, Camptothecin, Cis-Platinum, TPA, Nacl, Heat shock, Arsenic in TK6 (p53wt), NH32 (p53 KO) and MCF-7 breast cancer cells. In this experiments we concluded that GIF1 is not a stress inducible gene (Table 2).
- We found chemically synthesized siRNA for GIF1 is working (50% of KO), but to achieve more than 80% of KO, we constructed retrovirus expression siRNA for GIF-1 (Fig. 4).
- GIF1 was found as Gadd45-interacting proteins. To extend our study we performed another round of yeast hybrid screening and found GIF interact with two other nuclear hormone receptor, NUR77 and Androgen receptor (Table 1 in AM-2).
- GIF1 inhibits Nur77 transactivation activity (Fig. 3 in AM-2).
- Knockdown of the GIF1 protein increase transactivation of Nur77.

ROPORTABLE OUTCOME

Appended Manuscripts and Publications
(First year)
AM-1. Chung HK, Yi YW, Jung NC, Kim D, Suh JM, Kim H, Park KC, Song JH, Kim DW, Hwang ES, Yoon SH, Bae YS, Kim JM, Bae I, Shong M. CR6 Interacting Factor 1 (CRIF1) interacts with Gadd45 family proteins and modulates the cell cycle. 2003. April 25 (epub ahead of print).

(Second year)
AM-2 Park KC, Song KH, Chung HK, Kim H, Kim DW, Song JH, Hwnag ES, Chung H, Park SH, Bae I, Choi HS, Shong M. CR6 interacting Factor 1 (CRIF1) interacts with Orphan Nuclear Receptor Nur77 and inhibits its transactivation. Accepted in Mol. Endocrinology. 2004.

Reagent created:
(Second Year) In the course of these studies, we have created a varity of useful molecular reagents, including rAd-GIF1 (Figure) and retrovirus-siRNA for GIF1. In addtion, several GIF1 deletion mutant cDNA has been constructed.

Conclusions: We successfully constructed rAd-GIF1 and confirmed that exogenous GIF1 expression in breast cancer cell lines, MCF-10A and MDA-MB-231 cells (Fig. 1). We confirmed that overexpression of rAd-GIF arrested cells in G1 phase in human cell lines such as human skin fibroblast cells (HS27 and HS68) (Table 1). Currently we are examining the effects of rAd-GIF1 on breast cancer cell proliferation. To determine roles of GIF1 in stress response, we treated with a variety of stress-inducing agents shown in Table 2 and concluded that GIF1 mRNA is not stress inducible. Now we have a GIF1 Knockdown system working in MCF-10A cells (Fig. 4). In addition to our Task1-3, we found additional data on GIF1; GIF-1 interact with at least two nuclear hormone receptors; Androgen receptor and NUR77. Our studies indicate that GIF1 might act as a repressor of the orphan nuclear receptor Nur77 by inhibiting the AB domain-mediated transcriptional activities (AM-2).

Direction for future research:

The hindrance of this study was a poor transfection efficiency of GIF1 cDNA into human breast cancer cells. Now we have both overexpression and knockdown system for GIF1 working in our hands. We will confirm some of our preliminary data in breast cancer cell lines and then continue our study in Task3.

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Table 1

GIF1 caused cell cycle arrest in G1 in human cell lines. The rAd-GIF1 was infected into human skin fibroblast cells for 24hr and cell cycle profiles were analyzed by Facs analysis as described in Material and Methods in AM-1.

HS 27

	G1	S	G2/M
GFP-control	72%	16%	12%
rAd-GIF1	83%	6%	11%

HS 68

	G1	S	G2/M
GFP-control	85%	13%	2%
rAd-GIF1	93%	4%	3%

Table 2. Inducibility of GIF1 mRNA in response to stress.

Induction of GIF1 mRNA in response to environmental stress including X-ray, MMS, UV, H2O2 and etc. Cells were treated with a variety of stress inducing agents listed in Table 1. Semi-quantitative RT-PCR was used to determine expression of GIF1 in response to stresses. cDNA bands corresponding to amplified products were quantitated by densitometery and normalized by b-actin expression level. Raito between GIF1 mRNA expression from treated cell vs control was determined. Several cell lines with p53 wt or mutant were used for this study.

Stress source	TK6 (p53wt)	NH32 (p53 KO)	
8 Gy gamma	0.96	0.95	
50 ug/ml MMS	1.07	0.92	
100 J/m2 UVB	1.49		
200 uM H2O2	1.02	1.16	
200 ng/ml ADR	0.99	1.42	
2 uM Thapsigargin	0.96	1.42	
50 nM Taxol	1.11	1.27	
250 nM Camptothecin	1	0.96	
30 uM Cis-Platinum	1.01	0.96	
200 nM TPA	1.12	1.25	
500 mOsM NaCl	0.97	1.12	
Heat Shock	1.1	1.03	
30 uM Arsenic	0.89	1.15	
	A549	**MCF7**	**RKO**
20 Gy gamma	0.95	0.86	1.29
50 ug/ml MMS	1.22	0.68	1.27
14J/m2 UVC	1.02	1	0.78

Figure 1

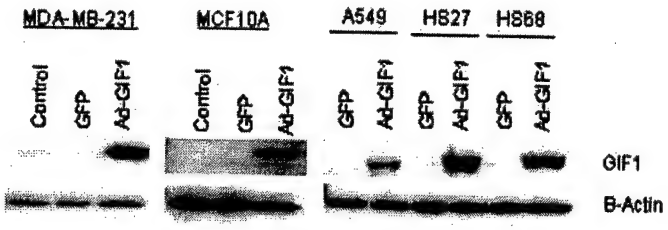

Figure 1. rAd-GIF1 or GFP-control adenovirus was infected into several human cell lines. GIF1 expression was determined by WB using anti-GIF1 antibody.

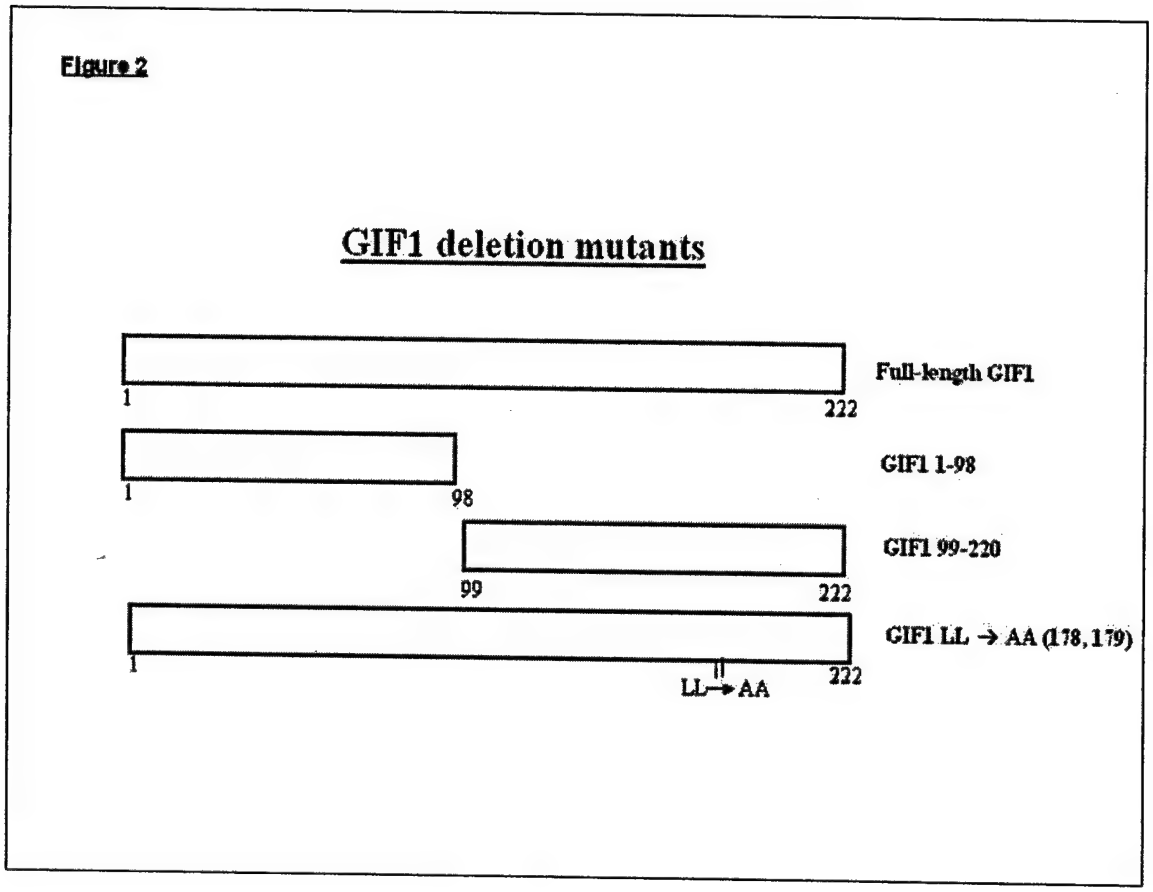

Figure 2. Construction of GIF1 deletion mutants. To map the binding site of Gadd45 on GIF1, we constructed deletion mutants of GIF1. Two deletion mutants, GIF1 N-terminal (1-98aa) and GIF1 C-terminal (99-220 aa) were constructed. GIF1 contain LXXLL motif in C-terminal region. We replaced LL with AA to construct point mutants.

Figure 3

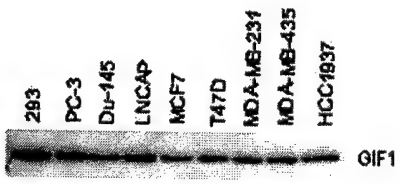

Figure 3. Expression level of GIF1 in human cell lines. In order to determine expression level of GIF1 in human cell lines, exponentially proliferating human cells were harvest for WB for GIF1. Compared to prostate cancer cell lines (PC-3, DU-145 and LNcaP) or human embryonic kidney cells (HEK 293), relative lower expression of GIF1 was found in several human breast cancer cell lines.

Figure 4

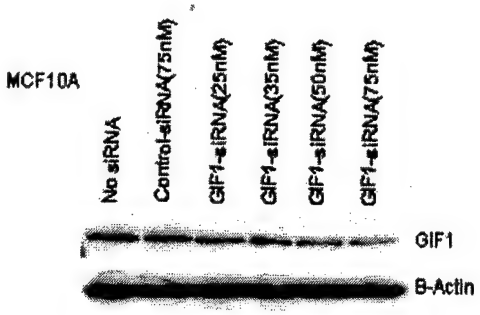

Figure 4. Treatment of siRNA for GIF1 in human breast cancer cells. Transfection condition and siRNA sequence information can be found in Material and Method in AM-1.

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CR6-interacting Factor 1 Interacts with Gadd45 Family Proteins and Modulates the Cell Cycle*

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Hyo Kyun Chung‡, Yong-Weon Yi‡§¶, Neon-Cheol Jung§¶, Daegun Kim§‖, Jae Mi Suh, Ho Kim, Ki Cheol Park, Jung Hun Song, Dong Wook Kim, Eun Suk Hwang, Soo-Hyun Yoon, Young-Seuk Bae**, Jin Man Kim‡‡, Insoo Bae§§¶¶, and Minho Shong‖‖**

*From the Laboratory of Endocrine Cell Biology, National Research Laboratory Program, Department of Internal Medicine, ‡‡Department of Pathology, Chungnam National University School of Medicine, Daejeon 301-721 Korea, §Therapeutic Gene Group, Team 1, Samyang Genex Biotech Research Institute, Yusung-Gu, Daejeon, the **Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea, the §§Department of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for Albert Einstein College of Medicine, New Hyde Park, New York 11040*

The Gadd45 family of proteins includes Gadd45α, MyD118/Gadd45β, and CR6/OIG37/Gadd45γ. These proteins play important roles in maintaining genomic stability and in regulating the cell cycle. This study reports the cloning of a novel protein called CR6-interacting factor 1 (CRIF1) which interacts with Gadd45α, MyD118/Gadd45β, and CR6/OIG37/Gadd45γ. CRIF1 binds specifically to the Gadd45 family proteins, as determined by an *in vitro* glutathione *S*-transferase pull-down assay and *an in vivo* mammalian cell two-hybrid assay along with coimmunoprecipitation assays. CRIF1 mRNA is highly expressed in the thyroid gland, heart, lymph nodes, trachea, and adrenal tissues. CRIF1 localizes exclusively to the nucleus and colocalizes with Gadd45γ. Recombinant CRIF1 inhibits the histone H1 kinase activity of immunoprecipitated Cdc2-cyclin B1 and Cdk2-cyclin E, and the inhibitory effects were additive with Gadd45 proteins. Overexpression of CRIF1 increases the percentage of cells in G_1, decreases the percentage of cells in S phase, and suppresses growth in NIH3T3 cells. The down-regulation of endogenous CRIF1 by the transfection of the small interfering RNA duplexes resulted in the inactivation of Rb by phosphorylation and decreased the G_1 phase cell populations. Expression of CRIF1 is barely detectable in adrenal adenoma and papillary thyroid cancer and much lower than in adjacent normal tissue. The results presented here suggest that CRIF1 is a novel nuclear protein that interacts with Gadd45 and may play a role in negative regulation of cell cycle progression and cell growth.

The growth arrest and DNA damage-inducible (Gadd45) family of genes includes Gadd45α (1), MyD118/Gadd45β (2), and CR6/OIG37/Gadd45γ (3–5). *Gadd45* was initially identified as a gene whose expression was rapidly induced by UV irradiation of Chinese hamster ovary cells (1). *MyD118* is an immediate early response gene induced by interleukin-6 in the murine myeloid cell line M1 (2). CR6/OIG37/Gadd45γ (5) is an activator of MEKK4/MTK1, an interleukin-2-induced immediate early gene (3), and an oncostatin M-inducible gene (OIG37) (4). The Gadd45 family members are highly conserved, with 68 and 70% amino acid sequence similarity between Gadd45γ and Gadd45α and Gadd45β, respectively.

Gadd45α is rapidly induced by agents that cause DNA damage including UV, *N*-acetoxy-2-acetylaminofluorene, methylmethane sulfonate, and H_2O_2 (6). DNA damage-induced transcription of the *Gadd45* gene is mediated by both p53-dependent (7) and p53-independent mechanisms (8). The phenotype of Gadd45-deficient mice is similar to the phenotype of p53-deficient mice, including genomic instability, sensitivity to radiation-induced carcinogenesis, and a low frequency of exencephaly (9). These findings suggest that Gadd45 is one component of the p53 pathway that maintains genomic stability.

Gadd45 proteins have also been implicated in DNA replication (10), DNA repair (10), G_1 progression and G_2/M checkpoint control (11, 12), apoptosis, and regulation of signal transduction (5, 13). Gadd45 proteins display a complex array of physical interactions with other proteins involved in these processes. It has been reported that Gadd45 proteins interact with proliferating cell nuclear antigen (10, 14–17, 20) and with cyclin-dependent kinase inhibitor p21 (17–20) in the nucleus. In addition, Gadd45 proteins activate the p38/Jun N-terminal kinase pathway by binding to MTK1/MEKK4 (5) in response to environmental stress. Gadd45 also interacts with Cdc2 and inhibits Cdc2-cyclin B1 kinase activity (21, 22). These findings led to the hypothesis that Gadd45 members play a critical role in negative growth

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF479749.

‡ The first two authors contributed equally to this work.

¶ Present address: Neurogenex Inc., Sillimdong 56-1 Kwanakku, Seoul 151-744 Korea.

‖ Present address: Dept. of Biological Science, Korea Advanced Institute of Science and Technology, Daejeon, 305-721, Korea.

¶¶ To whom correspondence may be addressed: Dept. of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for Albert Einstein College of Medicine, 270-05 76th Ave., New Hyde Park, NY 11040. Tel.: 718-470-7456; Fax: 718-470-9756; E-mail: insoobae@hotmail.com.

‖‖ To whom co-correspondence should be addressed: Laboratory of Endocrine Cell Biology, Department of Internal Medicine, Chungnam National University College of Medicine, 640 Daesadong Chungku, Daejeon 301-721 Korea. Tel.: 82-42-220-7161; Fax: 82-42-257-5753; E-mail: minhos@cnu.ac.kr.

A

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+1                                                                  ctg tga gga ccc cgc aca gcc aag
ATG GCG GCG TCC GTG CGA CAG GCA CGC AGC CTA CTA GGT GTG GCG GCG ACC CTG GCC CCG +60
 M   A   A   S   V   R   Q   A   R   S   L   L   G   V   A   A   T   L   A   P

GGT TCC CGT GGC TAC CGG GCG CGG CCG CCC CCG CGC CGC AGG CCG GGA CCC CGG TGG CCA +120
 G   S   R   G   Y   R   A   R   P   P   P   R   R   R   P   G   P   R   W   P

GAC CCC GAG GAC CTC CTG ACC CCG CGG TGG CAG CTG GGA CCG CGC TAC GCG GCT AAG CAG +180
 D   P   E   D   L   L   T   P   R   W   Q   L   G   P   R   Y   A   A   K   Q

TTC GCG CGT TAC GGC GCC GCC TCC GGG GTG GTC CCC GGT TCG TTA TGG CCG TCG CCG GAG +240
 F   A   R   Y   G   A   A   S   G   V   V   P   G   S   L   W   P   S   P   E

CAG CTG CGG GAG CTG GAG GCC GAA GAA CGC GAA TGG TAC CCG AGC CTG GCG ACC ATG CAG +300
 Q   L   R   E   L   E   A   E   E   R   E   W   Y   P   S   L   A   T   M   Q

GAG TCG CTG CGG GTG AAG CAG CTG GCC GAA GAG CAG AAG CGT CGG GAG AGG GAG CAG CAC +360
 E   S   L   R   V   K   Q   L   A   E   E   Q   K   R   R   E   R   E   Q   H

ATC GCA GAG TGC ATG GCC AAG ATG CCA CAG ATG ATT GTG AAC TGG CAG CAG CAG CAG CGG +420
 I   A   E   C   M   A   K   M   P   Q   M   I   V   N   W   Q   Q   Q   Q   R

GAG AAC TGG GAG AAG GCC CAG GCT GAC AAG GAG AGG AGG GCC CGA CTG CAG GCT GAG GCC +480
 E   N   W   E   K   A   Q   A   D   K   E   R   R   A   R   L   Q   A   E   A

CAG GAG CTC CTG GGC TAC CAG GTG GAC CCA AGG AGT GCC CGC TTC CAG GAG CTG CTC CAG +540
 Q   E   L   L   G   Y   Q   V   D   P   R   S   A   R   F   Q   E   L   L   Q

GAC CTA GAG AAG AAG GAG CGC AAG CGC CTC AAG GAG GAA AAA CAG AAA CGG AAG AAG GAG +600
 D   L   E   K   K   E   R   K   R   L   K   E   E   K   Q   K   R   K   K   E

GCG CGA GCT GCT GCA TTG GCT GCA GCT GTG GCT CAA GAC CCA GCA GCC TCT GGG GCA CCC +660
 A   R   A   A   A   L   A   A   A   V   A   Q   D   P   A   A   S   G   A   P

AGC TCC tga ggc ttt gtc cct tcc caa taa agc ctg cta cct ggc agt acc cct gaa gaa
 S   S
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B

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hCRIF1  M A A S V R Q A R S L L G V A A T L A P G S R G Y R A R P P P R R R P G P R W P D P E D L L T P R W 50
mCRIF1  M A A L A M R S G Y L L R L S V A L G P R S R S Y R A P P P P R R R P G P H S P D P E N L L T P R W

hCRIF1  Q L G P R Y A A K Q F A R Y G A A S G V V P G S L W P S P E Q L R E L E A E E R E W Y P S L A T M Q 100
mCRIF1  Q L T P R Y V A K Q F G R H G A I S G V P P A S L W P T P E Q L R E L E A E E Q E W Y P S L A T M Q

hCRIF1  E S L R V K Q L A E E Q K R R E R E Q H I A E C M A K M P Q M I V N W Q Q Q Q R E N W E K A Q A D K 150
mCRIF1  E S L R L Q Q Q A L E A R R Q A R E Q R I A E C M A K M P Q M I E N W R K Q K R E R W E K I Q A D K

hCRIF1  E R R A R L Q A E A Q E L L G Y Q V D P R S A R F Q E L L Q D L E K K E R K R L K E E K Q K R K K E 200
mCRIF1  E R R A R L Q A E A Q E R L G Y H V D P R S A R F Q E L L Q D L D K Q Q R K R L K E E R Q R Q K K E

hCRIF1  A R A A A L A A A V A Q D P A A S G A P S S 222
mCRIF1  A R I A A N A S A E A Q D S A V S G E P S S
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FIG. 1. **The nucleotide sequence and deduced amino acid sequence of human CRIF1.** *A*, the nucleotide and deduced amino acid sequence of human CRIF1. The *numbers* on the *right* indicate the nucleotide base pair position. The *underlined* nucleotide sequence is a putative nuclear localization signal. The CRIF1 nucleotide sequence was deposited in GenBank™ data bases with accession number AF479749. *B*, the deduced amino acid sequence of human CRIF1 and murine CRIF1. Identical amino acids are *boxed*.

control and apoptosis, but the detailed molecular mechanisms by which Gadd45 carries out this role remain to be elucidated.

This study used a yeast-two hybrid screen to identify novel proteins that bind to CR6/Gadd45γ. CR6-interacting factor 1 (CRIF1)[1] is a nuclear protein identified in this screen. CRIF1 interacts with Gadd45α, Gadd45β, and Gadd45γ, and it is ubiquitously expressed in human tissues. CRIF1 is exclusively localized to the nucleus and colocalizes with Gadd45γ. Gadd45 proteins and CRIF1 inhibit Cdc2-cyclin B1 kinase in an additive manner, and CRIF1 inhibits histone H1 kinase activity associated with immunoprecipitated Cdk2-cyclin E in the absence of Gadd45 proteins. Overexpression of CRIF1 inhibits cell cycle progression at G_1/S and suppresses growth in serum-stimulated NIH3T3 cells. In addition, adrenal adenoma and thyroid papillary cancer cells express CRIF1 at a very low level, which is lower than in adjacent normal tissue. These findings suggest that CRIF1 is a nuclear protein that modulates cell cycle progression and growth. Altered expression of CRIF1 may also contribute to tumorigenesis in some cell types.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—Dulbecco's modified Eagle's medium, Trizol, and LipofectAMINE were obtained from Invitrogen. Fetal bovine serum was purchased from Hyclone. Anti-FLAG antibody was purchased from Stratagene. The anti-Myc and anti-Rb (total and phosphospecific) antibodies were purchased from Cell Signaling Technologies, Inc. Horseradish peroxidase-linked anti-rabbit secondary antibody and chemiluminescent detection system were purchased from Cell Signaling Technologies. The Wizard Plus Miniprep DNA purification system and Plasmid Maxi kit were from Qiagen, and a vacuum manifold was purchased from Promega. All other enzymes and chemicals, either molecular biology grade or reagent grade materials, were purchased from Sigma.

The colon carcinoma cell line, RKO, the African green monkey cell line, COS-7, the mouse fibroblasts, NIH3T3, and the colon carcinoma cell line, HCT116, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 and 95% air atmosphere at 37 °C.

Plasmids—Plasmid DNA was generated using standard cloning procedures and constructs were verified by restriction enzyme analysis and

[1] The abbreviations used are: CRIF1, CR6-interacting factor 1; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TRITC, tetramethylrhodamine isothiocyanate.

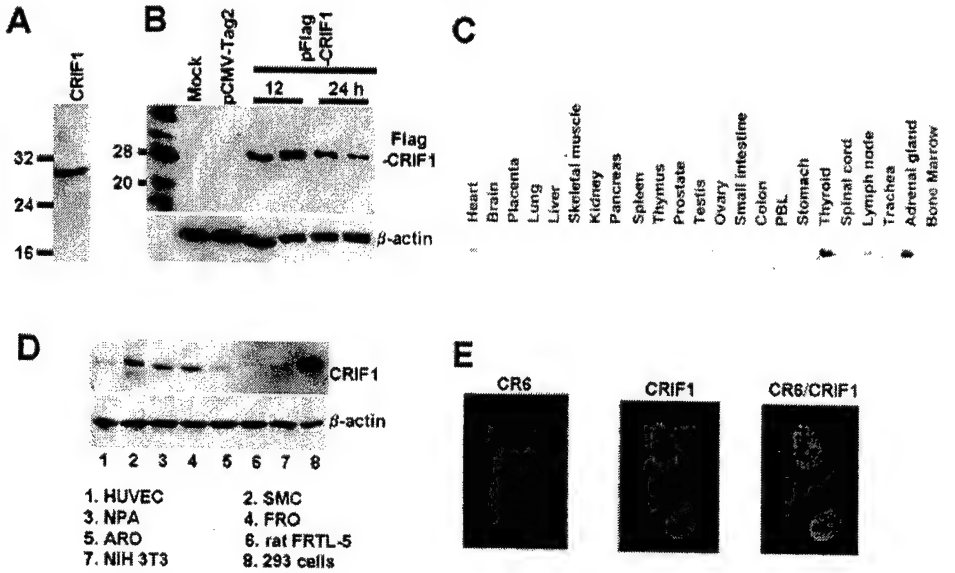

FIG. 2. **Expression and cellular localization of CRIF1.** *A*, the full-length CRIF1 coding sequence was synthesized by PCR with specific primers and inserted downstream of the T7 promoter into the pSP72 vector. *In vitro* transcription and translation were performed using a TNT-T7-coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine. A plasmid containing the luciferase gene was used as a control. The synthesized proteins were analyzed by 15% SDS-PAGE in the absence or presence of β-mercaptoethanol. *B*, the human CRIF1 full-length sequence was subcloned into the pCMV-Tag2 mammalian expression vector (Stratagene) downstream of the human cytomegalovirus promoter to generate pFLAG-CRIF1. pFLAG-CRIF1 or control pCMV-Tag2 was transfected into COS-7 cells by LipofectAMINE Plus. Proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis and Western blot with anti-FLAG antibody. *C*, the human mRNA blots were from Clontech Laboratories and included 2 μg of poly(A)$^+$ RNA/lane. CRIF1 cDNA or DECAprobe glyceraldehyde-3-phosphate dehydrogenase template (Ambion) was labeled by [α-^{32}P]dATP with the Strip-EZ™ DNA labeling kit (Ambion). *D*, Western blot with antiserum from New Zealand White rabbits immunized with CRIF1 peptide. Cell lysates from mouse, rat, and human cell lines were separated by 15% SDS-PAGE. *E*, COS-7 cells, transfected with pFLAG-CR6 and pMyc-CRIF1, grown on uncoated glass coverslips (Bellco) were incubated with anti-FLAG antibody and anti-c-Myc antibodies. Cells were washed five times with PBS+ and incubated with secondary antibody conjugated to TRITC or fluorescein isothiocyanate for 60 min at room temperature. Cells were washed five times in PBS+ before mounting. Fluorescence was visualized with a Nikon Eclipse E400 microscope equipped with epifluorescence optics and a digital camera; images were captured as Adobe Photoshop files.

DNA sequencing. Plasmid pAS-Gadd45γ encodes a fusion between the GAL4 DNA binding domain and Gadd45γ/CR6. pAS-Gadd45γ was constructed by inserting PCR-amplified human Gadd45γ cDNA into the pAS2–1 vector (Clontech Laboratories). Constructs for expression of GST fusion proteins were constructed by cloning into pGEX-4T (Amersham Biosciences). Human Gadd45α cDNA (supplied by Dr. A. J. Fornace Jr.) and PCR-amplified mouse Gadd45β/MyD118 and human Gadd45γ/CR6 cDNA were cloned into pGEX-4T.

For mammalian expression, pFLAG-Gadd45α, pFLAG-Gadd45β, pFLAG-Gadd45γ, and pFLAG-CRIF1 were constructed by inserting cDNA into the *Eco*RI/*Xho*I sites of pCMV-Tag2 (Stratagene). pMyc-CRIF1 was constructed by inserting cDNA into the *Eco*RI/*Xho*I sites of pCMV-Tag3 (Stratagene).

For mammalian two-hybrid assay, PCR-amplified Gadd45α, Gadd45β/MyD118, Gadd45γ/CR6, and CRIF1 cDNA were cloned into pCMV-BD and pCMV-AD (Stratagene).

Yeast Two-hybrid and cDNA Library Screening—Yeast strain Y190 (Clontech Laboratories, Inc.) was transformed with pAS-Gadd45γ and a Matchmaker two-hybrid cDNA library from K-562 cells (Clontech Laboratories, Inc.). Transformants were selected on SD medium lacking histidine, leucine, and tryptophan and containing 45 mM 3-amino-1,2,4-triazole (Sigma). After β-galactosidase filter assay, 72 blue colonies were obtained. To determine the specificity of interaction, pACT2 plasmid DNA was rescued from yeast, amplified in *E. coli*, HB101, and transformed into yeast Y190 with pAS-Gadd45γ. One of the clones (His$^+$ and β-gal$^+$) was sequenced and further analyzed. Full-length cDNA for CRIF1 was obtained from a human placenta cDNA library (Stratagene) as described previously (23).

In Vitro Interaction Experiments—*In vitro* translated ^{35}S-labeled CRIF1 was incubated for 20 min at room temperature with glutathione-Sepharose (10 μl) preloaded with GST-Gadd45α, GST-Gadd45β, GST-Gadd45γ, or GST-control in 250 μl of binding buffer (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin). Glutathione-Sepharose was washed extensively with binding buffer, and bound proteins were eluted in 25 μl of Laemmli sample buffer, boiled for 10 min, and analyzed by SDS-polyacrylamide gel electrophoresis (10%) followed by autoradiography.

Mammalian Two-hybrid Assay—Plasmids pCB-Gadd45γ (for GAL4 DBD-fusion) and pCA-CRIF1 (for NF-κB AD fusion) or pCB-CRIF1 and pCA-Gadd45γ (100 ng each) were co-transfected with 100 ng of pFR-Luc reporter (Stratagene) into NIH-3T3 cells and grown in 24-well plates using LipofectAMINE Plus (Invitrogen). Total plasmid DNA was adjusted to 400 ng/well with vector DNA (pCMV-BD or pCMV-AD). 100 ng of pCMV-β-gal DNA (Clontech Laboratories, Inc.) was used as internal control. Cells were lysed 24 h after infection, and luciferase activity was measured by Berthold LB9507 luminometer (Berthold). Luciferase activity was normalized to β-galactosidase activity.

Northern Blot Analysis—The human multiple tissue mRNA blots (Clontech Laboratories, Inc.) containing 2 μg of poly(A)$^+$ RNA per lane were hybridized using QuickHyb solution (Stratagene) under stringent conditions as recommended by the manufacturer. CRIF1 cDNA or DECAprobe glyceraldehyde-3-phosphate dehydrogenase template (Ambion) was labeled with [α-^{32}P]dATP with Strip-EZ™ DNA labeling kit (Ambion).

Immunoprecipitation and Western Blot Analysis—The following immunoprecipitation procedures were carried out at 4 °C using COS-7 cells coexpressing tagged Gadd45 α, β, and γ with/without pFLAG-CRIF1 transfection. Cells were grown on 100-mm dishes, washed with phosphate-buffered saline twice, and incubated for 30 min with radioimmune precipitation lysis buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was collected and centrifuged at 10,000 × *g* for 10 min. The supernatant was incubated with rocking for 2 h with 20 μl of protein A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and centrifuged for 15 min at 8,000 × *g*. The supernatant was removed and incubated with primary antibody for 2 h with rocking. Protein A/G beads were added, incubated for 12 h, and centrifuged at 10,000 × *g*. Immunoprecipitates were collected and washed three times with radioimmune precipitation buffer.

For Western blot analysis, cells were lysed at 4 °C for 20 min 10 mM KPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 2 mM dithiothreitol, 1% Nonidet p-40, 1 mM Pefabloc (Roche Applied Science), and 10 μg of each aprotinin and leupeptin per milliliter. Total protein lysates were denatured by boiling in Laemmli sam-

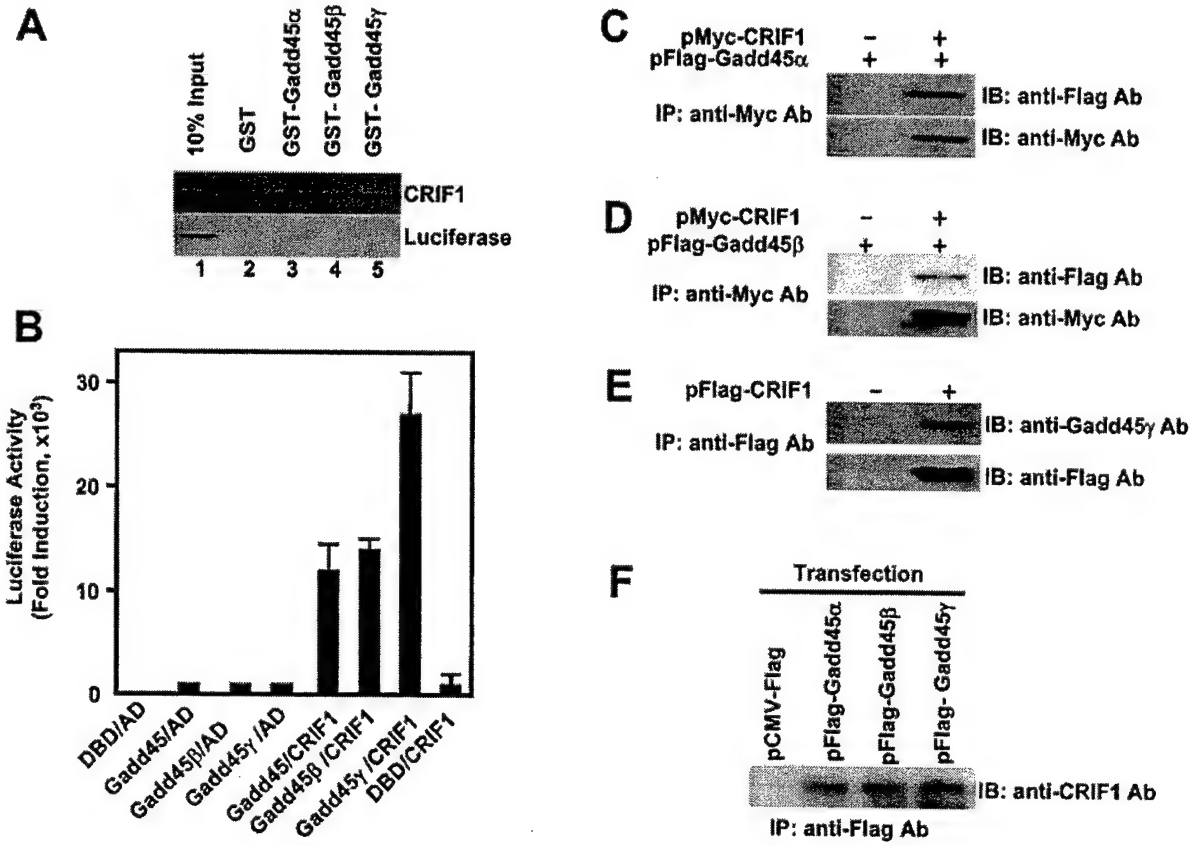

FIG. 3. **Interactions between CRIF1 and Gadd45 proteins.** *A*, *in vitro* translated ^{35}S-labeled CRIF1 was incubated for 20 min at room temperature with glutathione-Sepharose (10 μl) preloaded with GST-Gadd45α, GST-Gadd45β, GST-Gadd45γ, or GST control in 250 μl of binding buffer (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin). Beads were washed extensively with binding buffer, and bound proteins were eluted in 25 μl of Laemmli sample buffer, boiled for 10 min, and analyzed by SDS-polyacrylamide gel electrophoresis (10%) followed by autoradiography. *B*, pCB-Gadd45γ (for GAL4 DBD-fusion) and pCA-CRIF1 (for NF-κB AD fusion) or pCB-CRIF1 and pCA-Gadd45γ (100 ng each) were co-transfected with 100 ng of pFR-Luc reporter (Stratagene) into NIH-3T3 cells and grown in 24-well plates using LipofectAMINE Plus (Life Technologies). Total plasmid DNA was adjusted to 400 ng/well with pCMV-BD or pCMV-AD. 100 ng of pCMV-β-gal DNA (Clontech Laboratories) was used as internal control. 24 h after transfection, cells were lysed, and luciferase activity was measured by a Berthold LB9507 luminometer. Luciferase activity was normalized to β-galactosidase activity or protein. *C* and *D*, *in vivo* interactions between CRIF1 and Gadd45 proteins. COS-7 cells were transfected with tagged Gadd45α, -β, and -γ expression plasmids and CRIF1 expression plasmid. Immunoprecipitates containing CRIF1 were prepared with anti-FLAG and anti-Myc antibodies, and the immunoprecipitates were applied to a 15% polyacrylamide gel. Anti-FLAG (*C*), anti-Myc (*B*), and anti-Gadd45γ (*E*) were used for immunoblot analysis. *F*, COS-7 cells were transfected with pFLAG-Gadd45α, -β, and -γ expression plasmids, and immunoprecipitates were prepared by anti-FLAG antibody. Anti-CRIF1 antibody was used to detect coimmunoprecipitated endogenous CRIF1. *IB*, immunoblot; *IP*, immunoprecipitation.

ple buffer, resolved on 7.5–15% SDS-polyacrylamide gels, and transferred to polyvinylidene fluoride membranes. Membranes were blocked in PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween and incubated for 2 h with appropriate antibodies. Blots were developed using horseradish peroxidase-linked antirabbit secondary antibody and developed with a chemiluminescent detection system (Phototope-HRP Western blot detection kit; New England Biolabs).

Kinase Assay—Exponentially growing RKO cells were lysed on ice for 30 min in radioimmune precipitation buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). One mg of cellular protein was immunoprecipitated with anti-Cdc2 or Cdk2 antibodies. Immunoprecipitated Cdc2-cyclin B1 and Cdk2-cyclin E complexes were incubated with recombinant Gadd45α and Gadd45γ or CRIF1 proteins for 5 min. Histone H1 kinase assays were performed using 10 μg of histone H1 (Upstate Biotechnology, Inc., Lake Placid, NY), 15 mM $MgCl_2$, 7 mM β-glycerol phosphate, 1.5 mM EGTA, 0.25 mM sodium orthovanadate, 0.25 mM dithiothreitol, and 10 μCi of [γ-^{32}P]ATP in 25 μl. After 10 min at 37 °C, the reactions were transferred to numbered P81 paper and washed with 0.75% phosphoric acid and acetone. The activity in assay squares was measured by scintillation counting (Hewlett-Packard).

Flow Cytometry Analysis—Samples were prepared for flow cytometry essentially as described previously (24). Briefly, cells were washed with 1× phosphate-buffered saline and then fixed with ice-cold 70% ethanol. Samples were washed with 1× phosphate-buffered saline and stained with propidium iodide 60 μg/ml (Sigma) containing RNase 100 μg/ml (Sigma) for 30 min at 37 °C. Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer and Cell Quest version 1.2 software (Becton Dickinson Immunocytometry Systems, Mansfield, MA). At least 10,000 cells were analyzed per sample. Cell cycle distribution was quantified using the ModFit LT version 1.01 software (Verity Software House Inc., Topsham, ME).

Small Interfering RNA (siRNA) Experiments—The 21-nucleotide siRNA were synthesized and purified using a *Silencer* siRNA construction kit (Ambion Ltd.). The siRNA sequence targeting the human CRIF1 (GenBank™ accession number AF479749) corresponded to the coding region, as showed in Fig. 6*A*. The desalted DNA of the sense and antisense oligonucleotides targeting four different regions on the human CRIF1 (Fig. 6*A*) were synthesized, and the eight nucleotides at the 3′-end of both oligonucleotides had the following sequence: 5′-CCTGTCTC-3′, which is complementary to the T7 promoter. In order to produce an efficient transcription template, the sense and antisense oligonucleotides for each siRNA need to be converted to double-stranded DNA with a T7 promoter in 37 °C. The sense and antisense siRNA transcripts were transcribed for 2 h in separate reactions with T7 RNA polymerase. The reactions were then mixed, and the combined reaction was incubated overnight at 37 °C for double-stranded RNA. A single-strand specific ribonuclease and DNase digestion were used to eliminate the 5′-overhanging leader sequence and the DNA template,

respectively. The resulting siRNA was recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification. The cells were transiently transfected with the CRIF1-siRNA duplex and the expression plasmids using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. Briefly, the plasmids were mixed with the Plus reagent and then incubated with LipofectAMINE. The LipofectAMINE Plus DNA complex was added to the cells and further incubated at 37 °C for 3 h. The control cells received the LipofectAMINE Plus alone. The cell viability was detected using a trypan blue dye exclusion test. After incubation, the medium was removed, and the cells were given fresh medium and maintained for an additional 24 h. The transfection efficiency was evaluated with pCMV-SPORT-β-gal (500 ng) and pEGFP-CRIF1 (500 ng) in a six-well culture plate.

Immunofluorescence and Immunohistochemistry—COS-7 cells were transfected with pFLAG-Gadd45γ and pMyc-CRIF1 and grown on uncoated glass coverslips (Bellco Glass, Inc.). Cells were washed three times in PBS+ (PBS with 1 mM calcium and 1 mM magnesium). Cells were fixed with 3.7% formaldehyde for 15 min at room temperature, washed briefly in PBS+, and quenched with 50 mM NH_4Cl in PBS for 5 min. Cells were washed twice in PBS+ and incubated in blocking solution containing 3% bovine serum albumin in PBS+. For permeabilization, blocking and all subsequent incubation steps included 0.1% Triton X-100. Anti-FLAG antibody (Stratagene) and anti-c-Myc antibody (New England Biolabs, Inc., Beverly, MA) were incubated with the cells overnight at 4 °C. Cells were washed five times with PBS+ and incubated with secondary antibody conjugated with TRITC or fluorescein isothiocyanate for 60 min at room temperature. The cells were washed five times in PBS+, and fluorescence was visualized using a Nikon Eclipse E400 microscope equipped with epifluorescence optics and a digital camera; images were captured as Adobe Photoshop files.

Anti-CRIF1 antiserum was obtained from New Zealand White rabbits immunized with conjugated synthetic peptide AEEQKRREREQHI-AEC corresponding to the midregion of CRIF1. The sera were titered against the GST-CRIF1 fusion protein by enzyme-linked immunosorbent assay, and the serum exhibiting the highest titer (1:25,600) was used in subsequent experiments. Thyroid gland and adrenal gland tissues were obtained from surgery patients. Tissue specimens were fixed in 10% formalin, embedded in paraffin, and sectioned and then stained with hematoxylin and eosin to evaluate histologic type. Samples used in this study were confirmed histologically as benign or malignant.

CRIF1 expression was analyzed by immunohistochemistry in two adrenal gland and seven thyroid cancer tissues. Samples were microwaved for 10 min, and slides were incubated with anti-CRIF1 antiserum (1:800) at room temperature for 1 h. Preimmune sera did not stain tissues at a 1:800 dilution. Slides were washed in phosphate-buffered saline and incubated with a linking solution containing biotinylated goat anti-rabbit IgG (Dako LSAB kit; Dako Laboratories) at room temperature for 30 min. Slides were sequentially incubated with streptavidin peroxidase at room temperature for 30 min and 3,3′-diaminobenzidine chromogen substrate solution (Dako Laboratories), counterstained with Meyer's hematoxylin and mounted. Three independent readers evaluated the slides. Two independent readers graded the slides in a blinded fashion. The slides were graded with respect to staining intensity using the following scale: 0 (no staining), +1 (weak), +2 (moderate), and +3 (high).

Other Assays—Protein concentration was determined using the Bradford method (Bio-Rad) with recrystallized bovine serum albumin as a standard.

Statistical Significance—All experiments were repeated at least three times with different batches of cells. Values shown are the mean ± S.E. of three or more experiments. Significance between experimental values was determined by two-way analysis of variance.

RESULTS

Isolation, Expression, and Subcellular Localization of CRIF1—A yeast two-hybrid screen (25) was carried out to identify human proteins that interact with Gadd45γ. The screen used a Matchmaker two-hybrid cDNA library from K-562 cells (pACT2; Clontech Laboratories, Inc.) and a construct expressing a fusion between the GAL4 DNA binding domain and Gadd45γ. A partial cDNA was identified as a positive in this screen and called CR6-interacting factor 1 (CRIF1). Full-length human CRIF1 cDNA was isolated from a human placenta cDNA library (Stratagene) by hybridization (23).

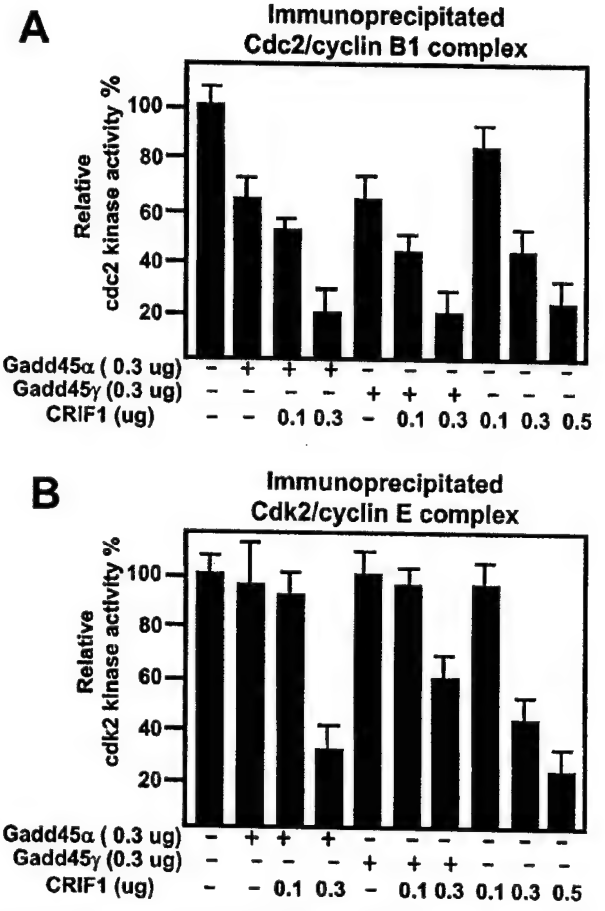

FIG. 4. **The effects of CRIF1 on Cdk2-cyclin E and Cdc2-cyclin B1 kinase activity with or without Gadd45α and -γ.** *A*, Cdc2-cyclin B1 complexes were immunoprecipitated from RKO cells, and the immunoprecipitates were incubated with the indicated amounts of recombinant Gadd45α, Gadd45γ, and CRIF1. Histone H1 kinase assays were performed as described. The activity was measured by scintillation counting (Hewlett-Packard). The results shown are the mean ± S.E. of three independent experiments. *B*, Cdk2-cyclin E complexes were immunoprecipitated from RKO cells and mixed with indicated amounts of recombinant Gadd45α, Gadd45γ, and CRIF1. Histone H1 kinase assays were performed as described. The activity was measured by scintillation counting (Hewlett-Packard). The results shown are mean ± S.E. of three independent experiments.

The CRIF1 cDNA had little 5′-untranslated sequence and a 669-bp open reading frame encoding a predicted polypeptide of 222 amino acids with a predicted molecular mass of 25 kDa (Fig. 1*A*). A polyadenylation signal (AATAA) is located 27 bp upstream of the poly(A) tail. A BLAST (26) search revealed that this cDNA encodes a novel protein with no homology to any proteins in the data base. The *CRIF1* gene is located on chromosome 19 at 19p13.2. The *CRIF1* gene has one exon of 351 bp and a second exon of 315 bp. Homology searches using BLAST identified the human and mouse genomic clones as BC004944 (similar to RIKEN cDNA 2310040G17) and NM026320 (mouse RIKEN cDNA 2310040G17), respectively. The amino acid sequence deduced from the CRIF1 cDNA isolated by two-hybrid screen is identical to BC004944 and has 90% homology with the mouse CRIF1 homologue NM026320 (Fig. 1*B*).

CRIF1 cDNA was *in vitro* translated, and the protein product had an apparent size of 28 kDa by SDS-PAGE (Fig. 2*A*). CRIF1 was also expressed transiently in COS-7 cells transfected with human CRIF1 cDNA cloned into pCMV-Tag2 (Stratagene). Cell

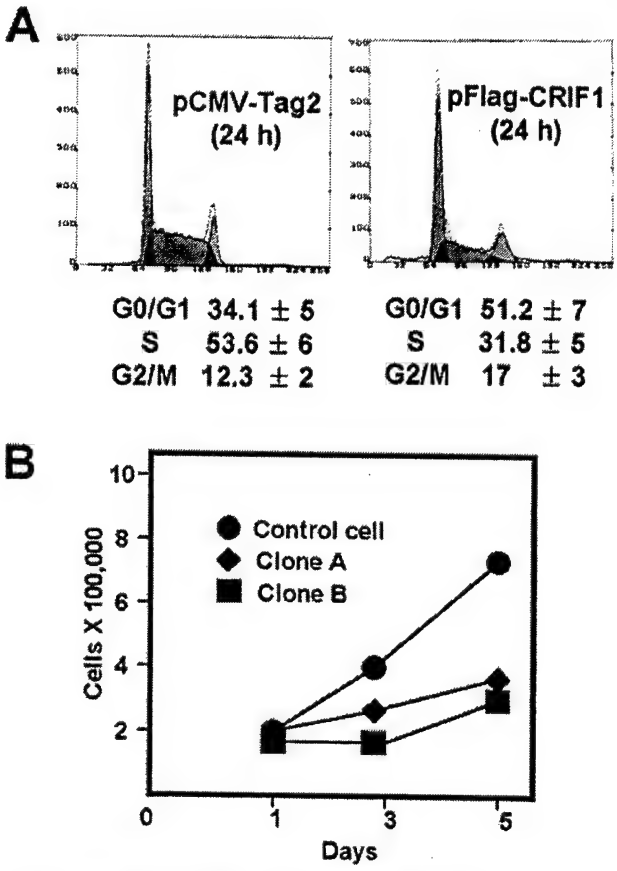

FIG. 5. **Effects of CRIF1 on cell cycle progression and cellular proliferation.** *A*, transfected cells were prepared as described under "Experimental Procedures." Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer and Cell Quest version 1.2 software (Becton Dickinson Immunocytometry Systems, Mansfield, MA). At least 10,000 cells were analyzed per sample, and cell cycle distribution was quantified using the ModFit LT version 1.01 software (Verity Software House Inc., Topsham, ME). *B*, NIH 3T3 cells were cotransfected with 5 μg of the indicated constructs and 1 μg of pCMV-puro and cultured in a puromycin-containing medium for 5 days. All assays were carried out in triplicate. A representative growth curve from the first set of clones is presented. *Closed circle*, control; *diamond*, clone A; *square*, clone B.

lysates were analyzed by Western blot using anti-FLAG antibody (Stratagene), and an immunoreactive band was detected of the expected size (Fig. 2*B*).

CRIF1 mRNA was examined by Northern blot using mRNA from cultured cells and human tissues (Clontech Laboratories). A CRIF1 mRNA of ~0.7 kb was detected in human cultured cells (data not shown). CRIF1 was highly expressed in heart, thyroid, lymph node, trachea, and adrenal tissue and was expressed at a low level in liver, skeletal muscle, kidney, pancreas, testis, ovary, and stomach (Fig. 2*C*). CRIF1 mRNA expression was similar to Gadd45γ (data not shown). Gadd45γ is induced by DNA-damaging agents including methylmethane sulfonate and UV (5). However, CRIF1 mRNA was not induced by ionizing radiation, methylmethane sulfonate, or UV in SW620 or MCF7 cells (data not shown).

Native CRIF1 was detected in human cells by Western blot analysis using anti-CRIF1 antiserum (Fig. 2*D*). The anti-CRIF1 antiserum specifically detects 28-kDa CRIF1 in anaplastic thyroid cancer cell lines NPA, FRO, and ARO and primary cultured smooth muscle cells and endothelial cells from umbilical vein (Fig. 2*D*). Immunoreactive CRIF1 protein was not detected in either the rat or mouse cells, such as the rat FRTL-5 thyroid cells and mouse NIH 3T3 cells (Fig. 2*D*).

Gadd45 proteins are localized predominantly in the nucleus (5, 10, 17). CRIF1 has a putative nuclear localization signal (28) in its C-terminal region (Fig. 1*A*), suggesting that it might colocalize with Gadd45. The subcellular location of CRIF1 and Gadd45γ was studied using COS-7 cells transfected with pMyc-CRIF1 and pFLAG-CR6. Myc and FLAG epitope tags were visualized by immunofluorescent staining (Fig. 2*E*). The results show that FLAG-CR6 protein and Myc-CRIF1 colocalize in the nucleus (Fig. 2*E*).

Interaction between CRIF1 and Gadd45 Proteins in Vitro and in Vivo—The interactions between CRIF1 and Gadd45 proteins were examined *in vitro* using recombinant GST-Gadd45α, Gadd45β, and Gadd45γ immobilized on glutathione-agarose beads and [^{35}S]methionine-labeled *in vitro* translated CRIF1. *In vitro* translated CRIF1 bound to GST-Gadd45γ but did not bind to GST (Fig. 3*A*, *lane 5 versus lane 2*). CRIF1 also binds to GST-Gadd45α and GST-Gadd45β (Fig. 3*A*, *lanes 3* and *4*). *In vitro* translated luciferase protein did not interact with GST or GST-Gadd45 proteins. As previously described (10, 14, 16, 17, 27), *in vitro* translated proliferating cell nuclear antigen and p21 bound to GST-Gadd45, Gadd45β, and Gadd45γ (data not shown).

CRIF1 interactions with Gadd45 proteins were also detected in mammalian cells using a mammalian cell two-hybrid assay (Fig. 3*B*). Gadd45α, Gadd45β, and Gadd45γ were cloned into pCMV-BD (Stratagene, La Jolla, CA) to create fusions with the DNA binding domain. CRIF1 was cloned into pCMV-AD (Stratagene) to fuse with the NF-κB activation domain. The luciferase reporter gene was activated when the CRIF1 fusion protein was co-expressed with Gadd45α, Gadd45β, or Gadd45γ fusion proteins (Fig. 3*B*). Similar results were obtained when CRIF1 was fused with the DNA binding domain and Gadd45 proteins were fused with the activation domain (data not shown). Co-expression of Gadd45α, Gadd45β, and Gadd45γ fusion constructs with vector pCMV-AD did not activate luciferase expression. This result indicates that CRIF1 interacts specifically with Gadd45 proteins.

In vivo interactions between CRIF1 and Gadd45 family proteins were examined using COS-7 cells co-expressing FLAG-tagged Gadd45α, -β, and -γ. CRIF1 was immunoprecipitated with anti-Myc and anti-FLAG antibodies and the precipitates were blotted with anti-FLAG or anti-Myc antibodies to detect tagged Gadd45 family proteins. As shown in Fig. 3, *C–E*, tagged Gadd45 family proteins were present in the CRIF1 immunoprecipitates. In addition, lysates from COS-7 cells expressing FLAG-tagged Gadd45α, -β, and -γ were immunoprecipitated with anti-FLAG antibody. CRIF1 was detected in the anti-FLAG immunoprecipitates (Fig. 3*F*). These results suggest that Gadd45 family proteins bind CRIF1 *in vitro* and *in vivo*.

Inhibition of Cdc2 and Cdk2 Kinase and Regulation of Cell Cycle by CRIF1—Gadd45 proteins regulate G_1/S and G_2/M checkpoints in several cell types (11, 18, 21, 22). Gadd45 proteins bind Cdc2 kinase, which inhibits cellular proliferation (11). Because these findings suggest that CRIF1 interacts with Gadd45 proteins *in vitro* and *in vivo*, Cdc2 kinase activity was examined in the presence or absence of Gadd45 proteins and CRIF1. As shown in Fig. 4, recombinant Gadd45α and Gadd45γ reduced histone H1 kinase activity in Cdc2-cyclin B1 immunoprecipitates to ~60% of the control (the presence of Cdc2-cyclin B1 was demonstrated by immunoblot; data not shown). The addition of recombinant CRIF1 resulted in a progressive inhibition of the enzymatic activity of the immunoprecipitated Cdc2-cyclin B1 complex. The simultaneous addition of recombinant Gadd45α or Gadd45γ and CRIF1 inhibited histone H1 kinase activity of Cdc2-cyclin B1 in an additive manner (Fig. 4*A*). In contrast, Gadd45 proteins do not inhibit Cdk2-cyclin E

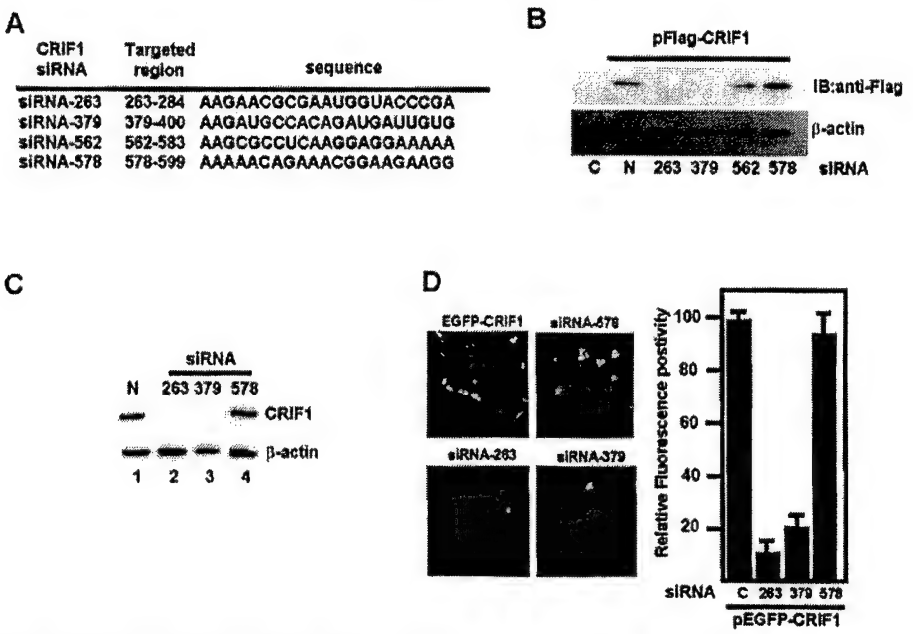

FIG. 6. **Knockdown of the CRIF1 protein.** *A*, CRIF-siRNA duplexes. The targeted region of CRIF1 is indicated with respect to the start codon (position 1). *B*, HCT116 cells were cultured in six-well plates until 80% confluence and transfected with either the CRIF1-siRNA duplexes (500 ng/well) or pFLAG-CRIF1 (500 ng/well) using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. After a 24-h transfection, the total lysates were prepared and blotted with anti-FLAG, anti-β-actin antibodies. The *figure* is representative of at least three separate experiments. *IB*, immunoblot. *C*, the HCT116 cells were cultured in six-well plates until 80% confluence and transfected with the CRIF1-siRNA duplexes (500 ng/well) using LipofectAMINE Plus (Invitrogen). After a 24-h transfection, the total lysates were prepared and blotted with anti-CRIF1 and anti-β-actin antibodies. The *figure* is representative of at least three separate experiments. *D*, the HCT116 cells were cultured in six-well plates until 80% confluence and transfected with the CRIF1-siRNA duplexes (500 ng/well) with pEGFP-CRIF1 (500 ng/ml) using LipofectAMINE Plus (Invitrogen). After 2 days of transfection, the cells on the coverslips were analyzed by fluorescent microscopy. The relative fluorescence positivity was calculated by counting the positive cells from five groups of 20–30 cells.

histone H1 kinase activity, and Gadd45 proteins have no effect on CRIF1-dependent inhibition of Cdk2-cyclin E histone kinase activity (Fig. 4*B*).

Because CRIF1 interacts with Gadd45 and inhibits the activity of Cdc2-cyclin B1 and Cdk2-cyclin E complexes, it is possible that CRIF1 could also influence cell cycle progression. This idea was tested by overexpressing CRIF1 in NIH 3T3 cells and monitoring cell cycle progression by flow cytometry. LipofectAMINE Plus was used to reproducibly transfect NIH3T3 cells with pFLAG-CRIF1 with an efficiency of 30–50% (Fig. 5*A*), eliminating the need for cotransfection with a selectable marker. Cell cycle progression was analyzed within 1–2 days after transfection. pFLAG-CRIF1 and pCMV-Tag2 (vector control) were transfected into NIH 3T3 cells, and CRIF1 expression was measured by immunoblotting with mouse anti-FLAG antibody. Cells transfected with pFLAG-CRIF1 expressed significantly more CRIF1 than pCMV-Tag2-transfected control cells (data not shown). Cells overexpressing CRIF1 (transfected with 4 μg of pFLAG-CRIF1) were more likely to be in G_1 and less likely to be in S phase than control cells (Fig. 5*A*).

The growth rate was also examined in cells overexpressing pFLAG-CRIF1. NIH3T3 cells were cotransfected with pCMV-Tag2 or pFLAG-CRIF1 and pCMV-puro and grown for 5 days; pCMV-puro encodes a gene that confers puromycin resistance for selection purposes during cell growth. Cells transfected with pFLAG-CRIF1 grew more slowly than control cells transfected with pCMV-Tag2 (Fig. 5*B*). After 5 days of selection, the number of cells overexpressing pFLAG-CRIF1 was about half the number of control cells.

Effects of CRIF1-siRNA Duplexes on Rb Phosphorylation and Regulation of Cell Cycle—In order to examine the role of endogenous CRIF1 in the regulation of cell proliferation and the cell cycle, 21-nucleotide duplexes of siRNA were used, and RNA interference studies in HCT116 cells were performed. This study selected four different target regions of human CRIF1 for the synthesis of the RNA duplexes as shown in Fig. 6*A*. The activities and specificity of the CRIF1-siRNAs was evaluated by the cotransfection of four different forms of siRNA duplexes with the expression plasmids, pFLAG-CRIF1 and pEGFP-CRIF1 (Fig. 6, *B* and *D*). As shown in Fig. 6*B*, two kinds of CRIF1-siRNAs, siRNA-263 and -379, effectively inhibited the exogenous FLAG-CRIF1 expression. However, siRNA-578 was not effective in inhibiting exogenous FLAG-CRIF1 expression. In similar experiments, siRNA-263 and siRNA-379 were effective in the reducing the green fluorescence protein fluorescence by inhibiting green fluorescence protein-tagged CRIF1 expression, as shown in Fig. 6*D*. However, siRNA-578 transfection did not alter the green fluorescence protein-CRIF1 fluorescence intensity compared with the control. These observations suggest that siRNA-263 and -379 are effective in inhibiting the exogenous CRIF1 expression. Therefore, the effects of siRNA-263 and -379 on the endogenous CRIF1 levels were investigated. The HCT116 cells transfected with CRIF1-siRNA-263 and -379 showed a very low level of endogenous CRIF1 compared with the nontransfected and siRNA-578-transfected cells (Fig. 6*C*). Collectively, these observations suggest that siRNA-263 and -379 are effective siRNA duplexes for the suppression of exogenous and endogenous CRIF1 expression.

During the G_1 phase, the growth factor-initiated signals promoted the accumulation and assembly of the D-type cyclins (D1, D2, D3) with their cognate cyclin-dependent kinases (CDK4 and -6). The active holoenzyme promoted the G_1 phase progression and S phase entry by virtue of its ability to phosphorylate the retinoblastoma protein Rb and titrate the CDK inhibitors $p27^{Kip1}$ and $p21^{Cip1}$. The initial phosphorylation of Rb and titration of the CDK inhibitors expedited the cyclin

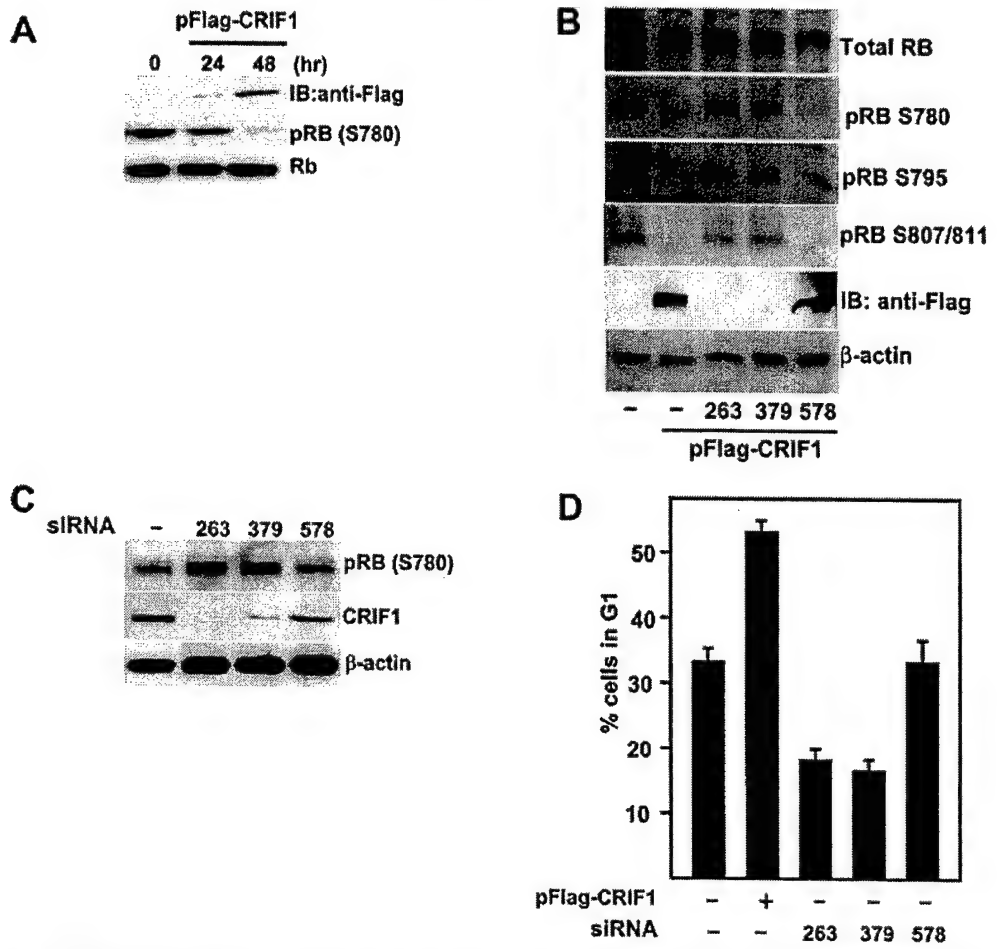

FIG. 7. **Effects of CRIF1-siRNA duplexes on phosphorylation of Rb.** *A*, HCT116 cells were cultured in six-well plates until 80% confluence and transfected with pFLAG-CRIF1 (500 ng/well). The whole cell lysates were separated by SDS-PAGE and immunoblotted (*IB*) with the antibodies to the total and phosphorylated Rb on the specific serine residue (Ser^{780}). The exogenous CRIF1 expression level was monitored by Western blot analysis using anti-FLAG antibodies. *B*, the HCT116 cells were cultured in six-well plates until 80% confluence and transfected with the pFLAG-CRIF1 (500 ng/well) and siRNA duplexes (500 ng/well). The whole cell lysates were separated by SDS-PAGE and immunoblotted with the antibodies to the total and phosphorylated Rb on the specific serine residues. The exogenous CRIF1 expression level was monitored by Western blot analysis using anti-FLAG antibodies. *C*, the HCT116 cells were cultured in six-well plates until 80% confluence and transfected with the siRNA duplexes (500 ng/well). Whole cell lysates were separated by SDS-PAGE and immunoblotted with the antibodies to total and phosphorylated Rb on the specific serine residue (Ser^{780}). The endogenous CRIF1 expression level was monitored by Western blot analysis using anti-CRIF1 antibodies. *D*, the HCT116 cells were cultured until 60% confluence, followed by mock or siRNA duplex transfection. After 48-h transfection, flow cytometric analysis was performed. The means and S.D. values of the percentage of cells in the G_1 phase for each of the treatments in the five independent experiments are shown. 15,000 cells were analyzed in each experiment.

E-CDK2 kinase activation, thereby completing the Rb phosphorylation and inactivation prior to the initiation of DNA replication. As described above, the exogenous CRIF1 overexpression decreased the Cdk2-cyclin E kinase activity. This study observed the role of CRIF1 in the phosphorylation of Rb by the overexpression and down-regulation of CRIF1. As shown in Fig. 7*A*, CRIF1 expression in the HCT116 cells resulted a decrease in the phosphorylated forms of Rb with an increase in the CRIF1 expression level. The decrease in phosphorylated Rb level by CRIF1 overexpression was completely inhibited by CRIF1-siRNA-263 and -379 cotransfection but not by CRIF1-siRNA-578, which was unable to suppress CRIF1 expression (Fig. 7*B*). The level of Rb phosphorylation after transfection of the siRNA duplexes, siRNA-263, -379, and -578 was analyzed in order to observe the effect of the down-regulation of the endogenous CRIF1 on Rb phosphorylation. The HCT116 cells transfected with siRNA-263 and -379 duplexes showed an increase in Rb phosphorylation, but siRNA-578 did not alter the endogenous CRIF1 and phosphorylated Rb levels (Fig. 7*C*). These siRNA experiments suggest that modulating the intracellular level of CRIF1 results in the alteration of the Rb phosphorylation status, which is involved in regulating the G_1/S phase of the cell cycle.

CRIF1 overexpression resulted in the accumulation of the G_1 phase populations as shown in Fig. 5*A*. This study analyzed the cell cycle in the cells transfected with the CRIF1-siRNA duplexes to observe the roles of the endogenous CRIF1 in regulating the cell cycle. As shown in Fig. 7*D*, CRIF1 overexpression resulted in increased G_1 phase populations, but the transfection of siRNA-263 and -379, which was highly effective in decreasing the endogenous CRIF1, resulted in a reduction of the G_1 phase cell populations. However, transfecting the inefficient siRNA duplex, siRNA-578, did not change the G_1 phase cell populations compared with the control. These observations suggest that the down-regulation of the endogenous CRIF1 promoted the cell cycle progression.

CRIF1 Expression in Endocrine Tumors—As shown in Fig. 2*C*, adrenal and thyroid gland tissue express CRIF1 at a high level. Therefore, the cellular distribution of CRIF1 in adrenal and thyroid glands was examined by immunohistochemistry in

normal and cancerous tissue from patients who underwent surgery for nonfunctioning adrenal tumor or papillary thyroid cancer. Deparaffinized adrenal adenoma (n = 2) and thyroid (n = 7) papillary cancer tissue was sequentially incubated with anti-CRIF1 antiserum (1:800), biotinylated secondary antibody, streptavidin peroxidase, and 3,3′-diaminobenzidine substrate. High level expression of CRIF1 was expected in adrenal and thyroid tissue based on previous Northern blot results (Fig. 2*C*). CRIF1 immunoreactivity was mainly confined to the cortex of the adrenal gland (*C*) (Fig. 8). Expression was highest in the zona glomerulosa region of the cortex, and CRIF1 expression was very low in adrenal medulla (*M*). CRIF1 immunoreactivity was noted at cytoplasm and nucleus. However, the adenoma (*A*) in the adrenal cortex (*C*) expressed CRIF1 at a significantly lower level than the surrounding tissue (Fig. 8). In the thyroid gland, CRIF1 immunoreactivity was high within the nucleus in follicular epithelial cells (Fig. 8); however, papillary cancer cells within the follicular epithelium had much lower CRIF1 immunoreactivity than the surrounding cells as shown in Fig. 8. Immunohistochemical findings from three patients are shown in Fig. 8; in addition, CRIF1 immunoreactivity was lower in seven papillary thyroid cancers than in normal thyroid follicular epithelial cells. The DNA sequences of exon 1 and exon 2 in CRIF1 in thyroid papillary cancer tissues from seven patients did not carry detectable mutations (data not shown).

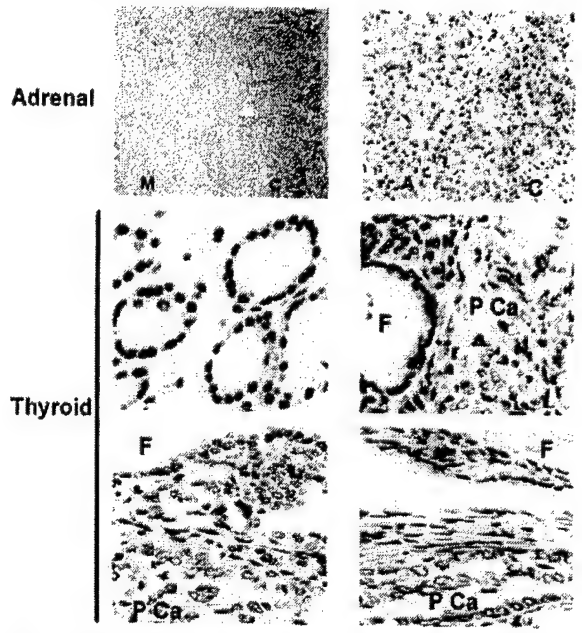

FIG. 8. **Immunohistochemistry of CRIF1 in normal and neoplastic tissues.** CRIF1 was localized in adrenal and thyroid tissue using polyclonal anti-CRIF1 antibody and a streptavidin peroxidase 3,3′-diaminobenzidine-chromogen detection system as described under "Experimental Procedures." *M*, adrenal medulla; *C*, adrenal cortex; *A*, adrenal cortical adenoma; *F*, thyroid follicle; *P Ca*, papillary thyroid cancer.

DISCUSSION

This study reports the identification of CRIF1, a novel nuclear protein that interacts with Gadd45 family proteins. Gadd45 plays several important biological functions including maintaining genomic stability and regulating cell cycle progression and apoptosis. Gadd45 interacts with several cell cycle regulatory proteins such as $p21^{WAF/CIP1}$ (17, 20), Cdc2 (21, 22), and proliferating cell nuclear antigen (10). MTK1/MEKK4 is a member of the mitogen-activated protein kinase kinase kinase family that also interacts with Gadd45 and activates Jun N-terminal kinase and p38 (5). These findings suggest that proteins that interact with Gadd45, including CRIF1, could play critical roles in regulating cell cycle progression.

A partial human CRIF1 cDNA was isolated using a yeast two-hybrid screen with Gadd45γ as bait. CRIF1 genomic sequence, chromosomal location, and a mouse CRIF1 homologue were identified by searching DNA databases. Evidence presented here indicates that CRIF1 interacts with Gadd45α, -β, and -γ proteins *in vitro* and *in vivo*. In addition, CRIF1 colocalizes with Gadd45γ in the nucleus. This result is consistent with the possibility that CRIF1 and Gadd45 proteins co-regulate nuclear functions such as cell cycle progression, replication, and transcription (29).

The function of Gadd45 proteins has been implicated in activating a G_2/M checkpoint in response to genotoxic stress agents such as UV radiation or methylmethane sulfonate. However, the function of Gadd45 proteins in negative growth control is not fully understood. Recently, it was shown that Gadd45 proteins α, β, and γ interact with and inhibit Cdc2-cyclin B1 kinase. This study shows that CRIF1 suppresses the histone H1 kinase activity of Cdc2-cyclin B1 and Cdk2-cyclin E complexes in the presence or absence of Gadd45 proteins. CRIF1 and Gadd45 inhibition was additive for Cdc2-cyclin B1 kinase but not for Cdk2-cyclin E kinase. These findings suggest that CRIF1 may modulate the G_1/S and G_2/M transition in dividing cells. Overexpression of CRIF1 alters cell cycle progression in NIH3T3 cells and increases the proportion of cells in G_1; thus, CRIF1 may participate in regulating the G_1/S transition, which is when cells commit to entering S phase and completing DNA replication and cell division. Small interfering RNA experiments revealed that the down-regulation of endogenous CRIF1 is related to the increase in Rb phosphorylation. This observation suggests that CRIF1 has a constitutive inhibitory role in G_1 cell cycle regulation.

The tumor suppressor, p53, is a key protein that regulates G_1 arrest in cells with DNA damage (30, 31). Genetic instability is one of the hallmarks of cancer, and cancer is often associated with aberrations in DNA repair or cell cycle checkpoint pathways (32). Proteins that regulate G_1/S, such as ATM (ataxia telangiectasia-mutated) (33), Chk2 (checkpoint kinase 2) (34), BRCA1 (35), p53 (31), p16 (36), and Rb (36), are frequently altered in cancer cells. These major G_1/S checkpoint transducers and effectors are tumor suppressors or protooncogenes, and loss of functional mutations in the genes encoding these proteins has been identified in many human malignancies.

CRIF1 expression is different in normal and cancer tissues. In normal tissue, thyroid and adrenal gland expressed the highest level of CRIF1 mRNA, and CRIF1 immunoreactivity is mainly localized in the nucleus by immunohistochemistry and *in vitro* immunofluorescence studies. However, histiocyte, osteoclast, terminally differentiated keratinocyte, and adrenal medulla expressed a very low or undetectable level of CRIF1 (data not shown), in contrast to high level CRIF1 expression in epithelial cells from thyroid follicle, colon (data not shown), and breast mammary duct (data not shown). It is interesting to note that some epithelial cell cancers in thyroid or breast expressed CRIF1 at a lower level than adjacent normal epithelial cells. Seven of seven papillary thyroid cancers expressed CRIF1 at a virtually undetectable level relative to normal epithelial cells and infiltrating T lymphocytes in the tumor, which expressed a high level of CRIF1. Reduced expression of CRIF1 was also observed in a benign adenoma from adrenal cortex. The mechanism by which CRIF1 expression is lowered in certain endocrine tumors was not evaluated in this study. No mutations that might account for altered expression of CRIF1 were found in exons 1 or 2 of CRIF1 in papillary thyroid cancer tissue.

In summary, the results presented here suggest that CRIF1 may negatively regulate cell growth by inhibiting the G_1/S transition. Reduced expression of CRIF1 may occur in some cancers, and altered expression of CRIF1 may play a significant role in carcinogenesis.

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CR6 Interacting Factor 1 (CRIF1) Interacts with Orphan Nuclear Receptor Nur77 and Inhibits its Transactivation

Ki Cheol Park, §Kwang-Hoon Song, Hyo Kyun Chung, Ho Kim, Dong Wook Kim, Jeong Hun Song, Eun Suk Hwang, Hyesook Chung, Su-Hyeon Park, §§Insoo Bae, §Hueng-Sik Choi, Minho Shong,

Laboratory of Endocrine Cell Biology, National Research Laboratory Program, Department of Internal Medicine, Chungnam National University College of Medicine, Deajeon 301-721 Korea. §Hormone Research Center, School of Biological Sicences and Technology, Chonnam National University, Kwangju, 500-757, Korea. §§Department of Oncology, Lombardi Cancer Center, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, DC 20057, USA.

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To whom Co-Correspondence to :

Minho Shong

Laboratory of Endocrine Cell Biology

Department of Internal Medicine

Chungnam National University College of Medicine

640 Daesadong Chungku Deajeon 301-721

Korea.
Tel) 82-42-220-7148
Fax)82-42-257-5753
Email) minhos@cnu.ac.kr

Hueng-Sik Choi
Hormone Research Center
Chonnam National University
Kwangju, 500-757 Korea
Tel) 82-62-530-0503
Fax) 82-62-530-0500
Email) hsc@chonnam.ac.kr

ABSTRACT

The CR6 interacting factor 1 (CRIF1) was recently identified as a nuclear protein that interacts with the Gadd45 (growth arrest and DNA damage inducible 45) family and participates in the regulation of the G1/S phase of the cell cycle. However, the nuclear action of CRIF1 is largely unknown. In this study, we demonstrate that CRIF1 acts as a novel co-regulator of orphan nuclear receptor Nur77 transactivation. Both in vitro and in vivo studies show that CRIF1 interacts with Nur77. The CRIF1 interacts with distinct AB domain of Nur77 and it dramatically inhibited the AB domain-mediated transactivation of Nur77. Transient transfection assay demonstrate that CRIF1 inhibit Nur77 transactivation and repress SRC-2-mediated Nur77 transactivation. The silencing of the endogenous CRIF1 by siRNA resulted in the activation of Nur77-mediated transactivation. The CRIF1 posses intrinsic repressor activities, but the repressive activities on Nur77 transactivation are not modified by histone deacetylase inhibitors, Trichostatin-A. In addition, over-expression of CRIF resulted in the inhibition of the hormone (TSH)/PKA-induced NurRE promoter activities. These results suggest that CRIF1 might act as a repressor of the orphan nuclear receptor Nur77 by inhibiting the AB domain-mediated transcriptional activities.

INTRODUCTION

The CR6 interacting factor 1 (CRIF1) has been identified as a protein that interacts with the Gadd45 (growth arrest and DNA damage inducible) family proteins, Gadd45α, Gadd45β, Gadd45γ (1). It has also been identified as a protein that interacts with the papilloma virus type 16 L2 protein (2). Although CRIF1 exhibits the ubiquitous expressions, high level of CRIF expression is observed in the endocrine organs, adrenal glands, thyroid, testes and ovaries (1). The studies on intracellular localizations of CRIF1 revealed that it is mainly expressed in the nucleus (1, 2). However, the intranuclear actions of CRIF1 have not been fully elucidated. Chung et al reported that CRIF1 inhibits the cyclin-dependent kinases, Cdc2/cyclin B1 kinase and Cdk2/cyclin E, so it renders inhibition of the cell cycle progression at the G1/S phase and suppresses growth in serum stimulated NIH3T3 cells (1).

The orphan nuclear receptor Nur77(NGFI-B) subfamily consists of three members including Nur77(NGFI-Bα), Nurr1(NGFI-Bβ) and Nor1(NGFI-Bγ) (4, 5, 6). Nur77 is widely expressed in the tissues including the thymus, muscle, liver, thyroid, lungs, testes, ovaries, ventral prostate, adrenal and pituitary glands(5, 6). The Nur77 subfamily members have been shown to bind DNA as monomers, homodimers, or heterodimers with RXR (7, 8, 9, 10). Recent advances have shown that the regulation of Nur77 occurs at transcriptional and post-translational levels. Nur77 has been shown to play an important role in regulating the expression of various genes in the hypothalamic-pituitary-adrenal axis (HPA) related to inflammation and steroidogenesis (11). In

particular, Nur77 expression is rapidly upregulated in the endocrine cells by stimulation of CRH, TSH, FSH, LH and ACTH (12, 13, ?).

Upon its expression, Nur77 may recruit its own interacting partners for either gene activation or repression. Although the transcriptional activity of many nuclear receptor dimmers is dependent on binding of a ligand to the c-terminal LBD, the molecular mechanism of ligand-induced transcriptional activity requires recruitment of coactivator protein to the activation domain. However, Nur77 is an orphan nuclear receptor lacking identified ligands and cofactors that regulate Nur77-dependent transactivation remains to be identified. In the recent works, coactivators and corepressors that regulate the Nur77 transcription activity have recently begun to be elucidated. For instance, The AF2-dependent coactivator ASC-2 and corepressor SMRT/NcoR have been shown to enhance or repress the Nur77 transactivation, respectively (14). Senali et al., showed that the N-terminal AB region which encoded the AF-1 domain was necessary for optimal Nur77-dependent transactivation in cultured C2C12 cells. In addition, they observed that SRC-2/TIF2/GRIP1/NCoA-2 modulate the activity N-terminal AF-1 domain of Nur77. Maira et al., also showed that p160/SRC coactivators are recruited to Nur77 dimers and that coactivator recruitment to the NurRE, the Nur-responsive elements is enhanced in response to corticotrophin releasing hormone (CRH) which activates protein kinase A. Moreover, PKA and the coactivator-induced potentiation of Nur77 activity are primarily exerted through the N-terminal AF-1 domain of Nur77 (15). These observations suggest that N-terminal AB region which contains AF-1 domain plays a major role in Nur77-mediated transactivation, coactivator

recruitment and endpoint effectors of hormone-induced PKA signaling pathways. This study identified the functional relationship between CRIF1 and the orphan nuclear receptor, Nur77. Both in vitro and in vivo studies show that CRIF1 interacts with Nur77. The silencing of the endogenous CRIF1 by siRNA resulted in the activation of Nur77-mediated transactivation. In addition, CRIF1 expression resulted in the inhibition of the hormone (TSH)/PKA-induced NurRE promoter activities. We propose that CRIF1 is a novel repressor of the orphan nuclear receptor, Nur77 and CRIF may play a key role in the regulation of TSH-mediated Nur77 transactivation.

RESULTS

Identification of the interaction between CRIF1 and Nur77. It has been reported that primary structure of CRIF1 showed coiled-coil region and nuclear localizing signals in the C-terminal half, and it exclusively localized within the nucleus. To identify the nuclear receptors which interact with CRIF1, we performed yeast two hybrid assay. Complementary DNA corresponding to the amino acid residues 1-222 (223 aa) of CRIF1 was fused to the LexA DNA binding domain (LexA-CRIF1) and used as bait in the yeast two hybrid screening tests. The LexA-fused full length CRIF1 and the B42-fused CRIF1, RXR, CAR, TR, SMRT and Nur77 were transformed into the *Saccharomyces cervisiae* EGY48s train containing β-glactosidase reporter plasmid 8H18-34 (Table 1). In addition, the LexA-fused ERRγ, GR, SHP, SF-1, mouse AR were also transformed into *Saccharomyces cervisiae* with the B42-fused CRIF1 (Table 1), and the transformants were selected on the plates with the appropriate selection markers. The β-galactosidase assay on the plates was performed in order to identify the interactions between CRIF1 and the nuclear receptors and the coregulators, SHP and SMRT/NcoR. The results of interactions between and CRIF1 and nuclear receptors and coregulators based on β-galactosidase assay were summarized in Table 1. The LexA-CRIF1 or B42-CRIF1 did not have any intrinsic activity of transcriptional activation for the reporters. The LexA-fused ERRγ, GR, SHP, and SF-1 did not show any significant interactions with B42-fused CRIF1 (Table 1). In addition, LexA-fused CRIF1 also did not have any significant reporter activity with B42-fused RXR, CAR, TR and SMRT. As demonstrated in Table 1, the LexA fusion proteins

including CRIF1 strongly interact with the B42 activation domain fused to Nur77. The mouse AR also had interactions with CRIF1 in the presence or absence of a ligand. However, its interactions between CRIF1 and AR were relatively weak compared with CRIF1 and Nur77.

The orphan nuclear receptor, Nur77, has conserved functional domains (16) as shown in Fig. 1A. B42-activation domain fusion constructs including full-length and deletion mutant Nur77 were created to investigate which domains of Nur77 interact with CRIF1. Interestingly, B42-Nur77 A/B domain interacted with CRIF1(Fig. 1B). However, B42-Nur77 CDE lost the interactions with the LexA-fused CRIF1. Moreover, a deletion of the AF2 region did not affect the interactions between CRIF1 and Nur77.. To further characterize the interactions between CRIF1 and Nur77, *in vitro* translated CRIF1 was prepared and performed pull-down assays with GST-Nur77 fusion protein. In agreement with the yeast two-hybrid results, CRIF1 interacted with A/B domain of Nur77. As a positive control experiment, Gadd45γ interacted with CRIF1 as shown in a previous study (1) (Fig. 1C). Taken together, these results suggest that CRIF1 interacts with a specific region, the AB domain of Nur77.

To observe the interaction between CRIF1 and Nur77 *in vivo*, the Human Skin fibroblast (HSF) cells were infected with adenovirus, AdCRIF1-GFP and AdGFP. As shown in Fig. 2A, HSF cell showed basal Nur77 expression (lower panel, Fig. 2A) and the infection of AdGFP and AdCRIF1-GFP expresses significant level of GFP and CRIF1 in protein (middle panel, Fig. 2A). The immunoprecipitates prepared by anti-GFP antibody contained the Nur77 (upper panel, Fig. 2A). This experiments shows that CRIF1 can interact with Nur77.

To observe the intracellular co-localizations of CRIF1 with Nur77, immunofluorescence confocal microscopy was performed on the NIH/3T3 cells co-transfected with pEGFP-CRIF1 and pCDNA3-HA-Nur77. The CRIF1 expression was mainly distributed in the nucleus (Fig. 2B). The rhodamine fluorescence, which indicates the distribution of the introduced HA-Nur77, was also exclusively localized in the nuclear compartment. CRIF1 co-localized with Nur77, as depicted in the merged image (Fig. 2B). Taken together, these results indicate that CRIF1 and Nur77 interacts with Nur77 *in vitro* and *in vivo*.

Inhibition of Nur77 transactivation by CRIF1. Previous studies have identified the NurRE and NBRE as a major binding sequences of Nur77 as dimer and monomer, respectively (10, 17, 18). To determine whether CRIF1 can modulate Nur77 transactivation, NurRE and NBRE driven reporters were cotransfected with Nur77 expression vector in presence or absence of CRIF1 in C2C12 cells. The activities of NurRE and NBRE reporters, a luciferase reporter plasmid driven by the transcriptional target of NGFI-B dimmers are strongly induced by expression of Nur77 (Fig. 3). Overexpression of CRIF1 repressed the Nur77-mediated reporter activities in a dose dependent manner (Fig. 3A, upper). However, CRIF1 expression did not affect the endogenous Nur77 level and did not show any specific effect on endogenous and exogenous Nur77 expression level, as shown in the lower panel of Fig. 3A. The NBRE-mediated transcription was also significantly reduced by CRIF1 (Fig. 3B). This result suggest that CRIF1 represses monomeric and dimeric Nur77-mediated transcriptional activities. Nur77 was fused to the Gal4-DBD, and the ability of

these chimeras to regulate the expression of the Gal4-TK-Luc reporter in C2C12 cells was examined (19). The Gal4-Nur77 chimera, encoding full length Nur77, activated transcription approximately by 14 fold (Fig. 3C). However, CRIF1 did not affect the reporter activity of Gal4-TK-Luc alone. The Gal4-Nur77-induced transcriptional activities were significantly inhibited by CRIF1 in a dose dependent manner. Increasing the amount of CRIF1 expression serially reduced the Gal4-Nur77 induced reporter activities (Fig. 3C). These observations suggest that CRIF1 represses the Nurr77 transactivation without affecting the level of Nur77 protein. Since CRIF1 interacted with the AB domain of Nur77 (Fig. 1), we examined the effects of CRIF1 on Nur77-AB domain-mediated transcriptional activities. Gal4-Nur77-AB increased the reporter activities approximately 10 fold. However, cotransfection of CRIF1 inhibited the Gal4-Nur77-AB domain-induced increase in the reporter activities in a dose dependent manner (Fig. 3D). However, Gal4-CDE did not increase the Gal4-TK-Luc reporter activities (data not shown). Overall, these observations indicate that CRIF1 may act as a repressor of Gal4-Nurr77 and the actions of CRIF1 inhibited the AB region of Nur77 contained a potent ligand-independent AF-1 domain.

The recruitment of coactivators in Nur77-mediated transcription still remained obscure. SRC-2 (GRIP-1) has been identified as a possible coactivator for the stimulation of Nur77 AB domain-mediated transcription (20). As shown in Fig. 4A, transfection of Nur77 expression vector efficiently induced NurRE transcription and this level of activity was stimulated significantly by 2-fold by addition of SRC-2 in C2C12 cells. However, the cotrasnfection of CRIF1

with SRC-2 resulted in the complete loss of SRC-2-mediated enhancement of NurRE activity. Furthermore, increasing amount of CRIF1 not only resulted suppression of SRC-2 effects but also further repressed the Nur-77-mediated tracriptional activities (Fig. 4A).

To determine whether CRIF1 has intrinsic repressor activities, transient transfection assays were performed to examine the repressor functions of CRIF1 in the basal transcription in C2C12 cells. When pCMX-CRIF1, which expresses Gal4-CRIF1 in-frame with the Gal4 DNA-binding domain (DBD), was co-transfected into the C2C12 cells with Gal4-TK-Luc, CRIF1 repressed the reporter gene expression in a dose-dependent manner (Fig 4B). The repressor activities of Gal4-CRIF1 were not influenced by treatment of HDAC inhibitor, Trichostatin A (Fig. 4B). Similary as shown in Fig. 4B, the TSA treatment (50 nM and 100 nM) did not affect the CRIF1 repressive activity on Gal4-Nur77 transcription (Fig. 4C). These observations suggest that the class I HDACs may not be involved in the CRIF1-mediated repression of the Nur77 transcriptional activities.

Determinatin of CRIF1 domain involved in the repression of Nur77 transactivation. To determine which region of CRIF1 were employed in the repression of Nur77, a series of CRIF1 deletion mutants were designed and prepared (Fig 5A). The primary structure of CRIF1 is schematically presented as in Fig.5A. The peptide sequence of CRIF1 was examined through the search of databases, BLAST, PSORT II, COILS, Pfam and TargetP. The region between amino acid residue 172 and 212 predicted as a coiled coil domains

and the region between the residue 184 and 200 contains the canonical nuclear localizing signal. The repressor activities of the CRIF1 deletion mutants were evaluated by measuring the repressive activities of Gal4-Nur77-AB-mediated transcription. The CRIF1 1-98, which has no mid-region and a C-terminal half of the wild type CRIF1, lost its repressor activities (Fig. 5B). However, CRIF1 1-171, which contains a N-terminal half and mid region, exhibited significant repressor activity. The deletion CRIF1 mutants, such as CRIF1 99-171 and CRIF1 99-222 which has a mid-region or a mid-region and c-terminal half, still exhibited strong repressor activity (Fig. 5B). These deletion mutant studies suggest that the mid-region of CRIF1 is involved in repressive function of Gal4-Nur77-AB-mediated transcription.

Effects of CRIF1 silencing on Nur77-mediated transcription. To investigate the role of endogenous CRIF1 on Nur77 transactivation, the Gal4-Nur77 with or without transfection of CRIF1 siRNA duplex were examined. We previously reported that siRNA-263 targeted 263~284 bp of the human CRIF1 cDNA was effective in the inhibition of endogenous CRIF1 expression (1). The 21-oligonucleotide duplex siRNA-263 effectively suppressed the expression of the exogenous and endogenous CRIF1 as shown in Fig. 6A. However siRNA-578 did not show any effect on silencing the endogenous and exogenous CRIF1 mRNA expression. As a result, siRNA-578 was used as the control siRNA for siRNA-263. The transcfection of CRIF1 siRNA-263 increased the Gal4-Nur77-mediated transcription about two fold in the absence of exogenous CRIF1. However, the control siRNA-578 did not affect the transcriptional

activities of Gal4-Nur77. Furthermore, the transfection of siRNA-263 inhibited the exogenous CRIF1-mediated repression of Gal4-Nur77 transcription while siRNA-578 did not affect the CRIF1-mediated suppression of the Gal4-Nur77 transcriptional activity. These observations suggest that the endogenous CRIF1 level may determine the Nur77 transcriptional activities. Since CRIF1 inhibited the Nur77-AB domain-mediated transcription (Fig. 3D), we examined the effects of siRNA-263 on the activity of the Gal4-Nur77-AB domain. Again, the transcfection of CRIF1 siRNA-263 increased the Gal4-Nur77-AB domain-mediated transcription in the absence of exogenous CRIF1 (Fig. 6B). The transfection of siRNA-263 also inhibited the exogenous CRIF1-mediated repression of Gal4-Nur77-AB transcription, whereas siRNA-578 did not affect the CRIF1-mediated suppression of Gal4-Nur77-AB transcriptional activity (Fig. 6B). Overall, the siRNA experiments showed that controlling the intracellular level of CRIF1 may determine the Nur77-mediated activity on gene transcription.

Repression of hormone- and PKA-mediated Nur77 transactivation by CRIF1. The thyroid stimulating hormones, TSH, are known to induce and activate Nur77 in target cells (13). To determine whether CRIF1 is involved in the TSH-mediated Nur77 transactivation, we examine the CRIF1 effects in TSH-induced increase of NurRE reporter activity. The FRTL-5 thyroid cells showed very low level of Nur77/NGFI-B mRNA, however, treatment of TSH resulted in strong induction of Nur77/NGFI-B mRNA (Fig 7A, upper right) within 1 h. TSH also shows induction of Nur77/NGFI-B protein (Fig 7A, upper left). As shown in Fig. 7A TSH increases the activities of NurRE reporter, which has

multiple Nur77 binding elements (Fig. 7A). However, CRIF1 expression decreased the TSH-induced NurRE reporter activities (Fig 7A) in a dose dependent manner.

Maira et al. reported that the CRH signals activate Nur77 through the cAMP/PKA pathway, and suggested that the PKA signaling pathways activate Nur77 via the AF1-dependent recruitment of coactivators (15). To determine whether CRIF1 is capable of modulating PKA mediated Nur77 transactivation, we cotransfect PKA expression vector with Gal4-Nur77. The expression of the catalytic PKA subunit increased the Gal4-Nur77 transactivation markedly as shown in Fig. 7B. However, the PKA-induced increase in the Gal4-Nur77 transactivation was significantly decreased by increasing the amount of CRIF1 expression. In a similar experiment, we observed the ability of CRIF1 in Gal4-Nur77-AB-mediated transactivation as shown (Fig. 7C). Again CRIF1 expression decreased the Gal4-Nur77-AB-mediated transactivation in a dose dependent manner. These observations suggest that CRIF1 repress the transcriptional activities of Nur77 by inhibiting the transactivating function of the AB domain (AF1) of Nur77 which are activated by TSH and PKA.

DISCUSSION

This study provides compelling evidence that CRIF1 interacts with the orphan nucleus receptor Nur77, and inhibits its transactivation. In particular, CRIF1 interacted with the AB (which encodes activation function -1, AF-1) domain of Nur77 and inhibit the AB domain-mediated transactivation. The Nur77 subfamily, Nur77, Nor-1 and Nurr1, shares common structural features, and they share similar a transactivation domain in the N-terminal portion, central DBD and C-terminal LBD. Although DBD and LBD are highly conserved, the N-terminal transactivation (AB) domain is not well conserved with a homology of 27% between Nur77 and Nurr1 or 21% between Nur77 and Nor1 (5, 6). Recent advances have shown that the N-terminal AB domain is involved in coactivator and kinase-induced transcriptional activation of Nur77 (15, 19). Moreover, the N-terminal AB region (not the LBD) facilitates the recruitment of coactivators such as SRC-2/GRIP-1 (15, 19). The results suggest that the AB domain plays a major role in the Nur77-mediated transcriptional activation and cofactor recruitment (15, 16). Interestingly, CRIF1 inhibits the Nur77 AB-domain-mediated transcriptional activities suggsting that CRIF1 competes with the coactivators that act on the AB domain of Nur77. However, it still remains to be elucidated whether CRIF interacts withSRCs, p300 and pCAF .

It was shown that Gal4-CRIF1 has intrinsic repressor activities. Furthermore CRIF1 repression of Gal4-Nur77 and Gal4-Nur77-AB domain-mediated transcription was not inhibited by the histone deacetylase inhibitors, Trichostatin A. Although these observations suggest that CRIF1 has an intrinsic repressor function, the domain that possess repressor activity has not been characterized.

The mid-region of CRIF1 can inhibit Gal4-Nur77-AB domain–mediated transcription. This region may have intrinsic repressor activities in CRIF1. Because the AB domain of Nur77 acts as a transcriptional activation domain by recruiting the transcriptional coactivators, CRIF1 may also act as a competitor for those coactivators.

The N terminus (AB domain) of Nur77 was also shown to be hyperphosphorylated in response to variety of signals, and such actions would be consistent with the involvement of AF-1 domain in PKA action (15). The serine/threonine-rich domains in the N-terminus have been implicated in the regulation of Nur77-dependent transcription (23, 24). The role of phosphorylation in the AB domain of Nur77 pontiates the AF-1-mediated transactivation by recruitment of coactivators. As shown in Fig. 7, the catalytic subunit of PKA ncreases the Gal4-Nur77 and Gal4-Nur77-AB-mediated transcription, but the PKA effects are inhibited by CRIF1. Several hypothalamic and pituitary hormones for example, CRH, LH and TSH activate the PKA system, and are involved in the induction and activation of Nur77 (13, 15, 25, 26). The findings that CRIF1 expression modulates the TSH effects on NurRE activity suggests CRIF1 may be involved in the hormone-mediated regulation of Nur77 transcriptional activity.

Chung et al reported that CRIF1 is involved in regulating the cell cycle, it acts as a negative regulator in the G1 to S phase progression by inhibiting the cyclin-dependent kinases (1). The relationships between the regulation of the cell cycle and the repression of the Nur77 activities are still remains to be elucidated. Recently, Nur77 has been demonstrated to be a molecule involved

in the apoptotic process. Nur77 translocates from the nucleus to the cytoplasm where it targets the mitochondria to induce the release of cytochrome *c* and apoptosis (27). However, CRIF1 may not modify the apoptotic responses caused by Nur77 because the apoptotic effect of Nur77 does not require its transcriptional activity or DNA binding. Nur77, which is induced by growth factors, EGF, and serum, is involved in the cell cycle progression and proliferation (28). The mitogenic effect of Nur77 requires its DNA binding and transactivation functions. From these observations, it is possible to speculate that the inhibition of Nur77-mediated transcription is one possible mechanism for inhibition of the cell cycle progression by CRIF1.

MATERIALS AND METHODS

Reagents

Highly purified bovine TSH and Trichostatin A (TSA) and all other materials were obtained Sigma-Aldrich (St. Louis, MO).

Plasmids: cDNA, reporter, deletion construct of CRIF, pEGF-CRIF등를 describe하고 DNA를 얻은 것은 origin을 언급할 것)

Yeast two-hybrid protein interaction assay

The interaction between CRIF1 and Nur77 in the yeast was measured by activating the lacZ reporter constructs, as detected by β-gal assays . Yeast strain, EGY48 (p80p-lacZ) (CLONTECH Laboratories, Inc., Palo Alto, CA) was transformed with the appropriate plasmids encoding the Lex A- DBD fusion of CRIF1, ERRγ, mAR, GR, SHP and SF-1 with the plasmids encoding the B42-AD fusion of CRIF1, RXR, CAR, TR, SMRT and Nur77 proteins. The colonies were selected in the synthetic medium lacking l-Uralic, l-Histidine, and l-Tryptophan (SC-UHW) at 30 °C for 3 days, and the -gal activity in the extracts prepared from the liquid culture was determined. Five independent colonies from each plate were grown overnight in 2 ml SC-UHW with or without the indicated concentration of BPA. The cells were the harvested and assayed for the -gal activity as described previously.

Cell Culture (yeast two-hybrid다음 에 넣으면 좋겠음)

The African green monkey cell line, COS-7, the mouse fibroblasts, NIH3T3, Human Skin fibroblast ,HSF and C2C12 mouse skeletal muscle cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, inc.) supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, nonessential amino acids, 100 units/ml penicillin, and 100 g/ml streptomycin in humidified 5% CO_2 and 95% air at 37℃.

FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD) were a fresh subclone (F1) that had all the properties previously detailed (29). Their doubling time with TSH was 36 ± 6 h, and they did not proliferate without TSH. After 6 days in the medium with no TSH, the addition of 1 x 10-10 M TSH stimulated the thymidine incorporation into the DNA at least 10 fold. The cells were diploid between their 5th and 20th passages. The cells were grown in a six-hormone medium consisting of Coon's modified F-12 supplemented with 5% calf serum, 1 mM nonessential amino acids, and a mixture of six hormones (6H): bovine TSH (1 μ/ml), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Fresh medium was added to all the cells every 2 or 3 days, and the cells were passaged every 7 or 10 days. In individual experiments, the cells were shifted to 5H medium with no TSH and 5% calf serum.

In Vitro Translation

CRIF1 was transcribed and translated *in vitro* by using a coupled rabbit reticulocyte system (TNT, Promega) in the presence or absence of [^{35}S] methionine (Amersham Bioscience, Inc.) according to the manufacturer's

protocol.

GST Pull-down Assay

A GST pull-down assay was performed according to the method described previously (1). Briefly the GST fusion proteins or GST protein only were expressed in the Escherichia coli BL21 (DE3) pLys bacterial culture, which were recovered on glutathione-Sepharose-4B beads (Amersham Bioscience, Inc.), The GST fusion proteins were analyzed on 10% SDS-polyacrylamide gels for their integrity and to normalize the amount of each protein. The Promega TNT-coupled transcription-translation system was used to produce [35S] methionine labeled CRIF1, which were visualized by SDSPAGE. The *in vitro* binding assays were performed with glutathione-agarose beads Amersham Bioscience, Inc.) coated with 500 ng of the GST fusion protein and 2–10 μl of [35S]methionine-labeled protein in 200 μl of a binding buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 5 μg of ethidium bromide, and 100 μg of bovine serum albumin. The reaction was allowed to proceed for 1–2 h at 4 °C with constant rocking. The affinity beads were then collected by centrifugation and washed five times with 1 ml of the binding buffer without ethidium bromide and bovine serum albumin. The beads were resuspended in 20 μl of the SDS-PAGE sample buffer and boiled for 5 min. The eluted proteins were fractionated by SDS-PAGE, and the gel was treated with Amersham Amplify fluor, dried at 70 °C, and autoradiographed.

Immunoblotting

The cells were lysed by adding the SDS sample buffer (62.5 mM Tris-Hcl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 125 mM DTT, and 0.03% (w/v) bromophenol blue). The total cell lysates were denatured by boiling for 5 min, resolved on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in TBS containing 5% (wt/vol) milk and 0.1% Tween for 1 h, and incubated for 2 h with the primary antibodies. The blot was developed using the HRT-conjugated secondary antibodies (Phototope-HRP Western Blot Detection Kit, New England Biolabs, Beverly, MA).

Immunoprecipitations

The following immunoprecipitation procedures were carried out at 4 C. Cells grown on 100-mm dishes were washed with PBS twice before lysis. Radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (20 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml chymostatin, 2 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) was added for cell lysis and incubated for 30 min. The cell lysate was collected, triturated, and centrifuged at 1000 x *g* for 10 min. To preclear the cell lysate, the supernatant was mixed with 20 µl of protein A/G beads (Santa Cruz Biotechnology, Inc.), incubated for 30 min while rocking, and centrifuged for 15 min at 1000 x *g*. Precleared samples were incubated with a primary antibody for 2 h with rocking, and then protein A

beads were added, incubated for 1 h, and centrifuged at 1000 x *g*. The immunoprecipitates were collected and washed three times with RIPA buffer.

Adenovirus CRIF construction????

Confocal Microscopy

The NIH/3T3 cells were grown on coverslips and transfected with pEGFP-CRIF1 and pCDNA3-HA-Nur77 using the LipofectAMINE method (Invitrogen) (30). The quiescent cells were washed three times with cold phosphate-buffered saline and fixed in 3.7% formaldehyde for 40 min. The fixed cells were mounted on glass slides with phosphate-buffered saline and observed using laser-scanning Confocal microscopy (Olympus). In order to detect the pCDNA3-HA-Nur77, the cells mounted on glass slides were permealized with 2 ml PBS containing 0.1% Triton X-100 and 0.1M Glycine at room temperature, incubated for 15 min, washed three times with 1x PBS and blocked with 3% (w/v) BSA in PBS for 10 min at RT. The cells were incubated with the primary anti-HA antibody for 1 h at 37°C, washed three times with 1x PBS and incubated for 1 h with the Rhodamine-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch) at 37°C.

Transient transfection and luciferase assay

The pNBRE3-tk-Lluc promoter and Gal4-TK-Luc promoter constructs were transfected in either the FRTL-5 thyroid cells or the C2C12 mouse skeletal muscle cells by lipofection with a Lipofectamine regent (Invitrogen, San Diego, CA). For each well of the 12-well plate, 4 µl of Lipofectamine were combined

with 200µl of OptiMEM (Life Technologies/BRL), which were then added to 0.2 µg of the pNBRE3-tk-Luc promoter and Gal4-TK-Luc promoter construct. The cells were incubated overnight with a DNA/Lipofectamine mixture. Either the 5H5% medium or Dulbecco's modified Eagle's medium was applied, and the cells were incubated for an additional 24 h prior to determining the luciferase activity.

The cells were washed with PBS and lysed with 180 µl of a lysis buffer. The cells were cotransfected with 0.1µg of the pRL-CMV plasmid containing the *Renilla* luciferase gene (Promega, Madison WI) according to the manufacturer s protocol. The extracts were assayed for their luciferase activity in triplicate, and the light intensity was measured using a luminometer (Berthold, Bad Wildbad, Germany). The luciferase activity was integrated over a 10-s period. The firefly luciferase values were standardized to the *Renilla* values.

Small Interfering RNA (siRNA) Experiments

The 21-nucleotide siRNA were synthesized and purified using a Silencer siRNA construction kit (Ambion Ltd.). The siRNA sequence targeting the human CRIF1 (GenBankTM accession number AF479749) corresponded to the coding region.(1) The desalted DNA of the sense and antisense oligonucleotides targeting the four different regions on the human CRIF1 were synthesized, and the eight nucleotides at the 3-end of both oligonucleotides had the following sequence: 5-CCTGTCTC-3, which is complementary to the T7 promoter. In order to produce an efficient transcription template, the sense and antisense oligonucleotides for each siRNA needed to be converted to the double stranded

DNA with a T7 promoter at 37 °C. The sense and antisense siRNA transcripts were transcribed for 2 h in separate reactions with T7 RNA polymerase. The reactions were then mixed, and the combined reaction was incubated overnight at 37 °C for the double-stranded RNA. A single-strand specific ribonuclease and DNase digestion procedure was used to eliminate the 5-overhanging leader sequence and the DNA template, respectively. The resulting siRNA was recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification.

Northern blot analysis

Total cellular RNA was isolated using the standard procedures, and Northern analysis was performed as previously described (21). The final washes were conducted at 65°C in 1_ SSPE (150 mM NaCl, 10 mM NaH2PO4, and 1 mM EDTA, pH 7.4). The hybridization probes for Nur77 was the purified inserts of the expression vectors pcDNA3-Nur77 respectively. The probe was radiolabeled using a random priming protocol (Amersham Pharmacia Biotech, Arlington Heights, IL).

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FIGURE LEGENDS

Figure 1. CRIF1 Interacts with the AB domain of Nur77. (A) Schematic representation of the Nur77 wild type and deletion mutants. **(B)** Plasmids encoding LexA-CRIF1 were cotransformed with the plasmids encoding B42-AD fusion of the Nur77 and Nur77 deletion mutants AB, CDE, ΔAF2 were transformed into the yeast strain, EGY28. The β-gal activity was measured as described in Materials and Method. **(C)** Glutathione-S-transferase pulldown assay, GST, GST-Nur77, GST-Nur77AB, and GST-Gadd45α were incubated independently with ^{35}S-radiolabeled full-length CRIF1 *in vitro*. The *input lane* represents 10% of the total *in vitro* transcribed and translated protein.

Figure 2. Association of CRIF1 and Nur77 *in vivo.* (A) Human Skin fibroblast (HSF) cell lines were infected with Ad-CRIF1 or Ad-GFP at the MOI indicated. CRIF1-GFP was immunoprecipitated with anti-Nur77 antibody. The immunoprecipitated proteins were analysed by Western blotting using anti-GFP antibody. And total cell lysates were prepared and subjected to Western blot analysis in SDS-PAGE with the GFP and Nur77 antibody. **(B)** Subcellular Colocalization between CRIF1 with Nur77. NIH3T3 cells were transiently transfected with pEGFP-CRIF1 (1 μg/dish) and HA-Nur77 (1 μg/dish) by lipofectAMINE (Invitrogen). At 24 h after transfection, the cells were fixed in 4% formaldehyde for 30 min. The fixed cells were mounted onto glass slides with PBS and observed with a laser-scanning Confocal microscope. The GFP-fused wild-type CRIF1 were detected by autofluorescence, and Nur77 were detected by staining with the primary monoclonal anti-HA antibody and the Rhodamine-

conjugated secondary antibody. The yellow stain in the merged image depicts the colocalization of CRIF1 and Nur77. The figure shows representative cells from one of three independent experiments.

Figure 3. Inhibition of Nur77 Transactivation by CRIF1. C2C12 skeletal muscle cells were cultured in 12 well plates until they reached 80% confluence and the left panel figure were cotransfected with 0.2μg of the **(A)** NurRE or **(B)** NBRE reporter construct with the addition of 0.1μg of pCDNA 3.1-Nur77, 0.01-0.2μg of CRIF1. and the right panel figure were 0.2μg Gal4-Tk-Luc reporter construct and with or without **(C)** 0.1μg of Gal4- Nur77, **(D)** 0.1μg of Gal4-Nur77-AB, 0.01-0.2μg of CRIF1 added, After 24-h transfection, the cells were lysed, and the luciferase activity was measured by a Berthold LB9507 luminometer. The luciferase activity was normalized by *Renilla.* The fold activation was expressed relative to the luciferase activity obtained after cotransfection of the pCMV alone. The histogram represents the average of three independent sets of transfection experiments with the error bars indicating one s.d. The lower panel of (A) shows the Western blots analysis of Nur77 and CRIF1 expression in cells cotransfected with the indicated expression plasmids. after 24hr incubation; cells were harvested and Western blot with SDS-PAGE on 10% gel and immunoblotted with anti-Nur77, anti-Flag and anti-actin.

Figure 4. co-activator, repressor can not potentiate the activity of the Nur77 by CRIF1 (CRIF은 SRC-2에 의한 Nur77 transactivation을 inhibiton하고 TSA는 effectrk 없다라고 해야할 것 같음). The C2C12 skeletal

muscle cell cultured 12 well plate and cotransfected with 0.2μg of the NurRE reporter construct and **(A)** 0.1μg of the Nur77, 1μg of the SRC-2 and 0.01-0.2μg of the CRIF1. also same prepared cell were cotransfected with 0.2μg of the Gal4-Tk-Luc reporter construct and with or without 0.1-0.2μg of Gal4-CRIF1 **(B)** and treated with 100nM Trichostatin A (TSA) or DMSO. **(C)** 0.1μg of Gal4- Nur77, 0.05-0.1μg of CRIF1 and treated with 50, 100nM Trichostatin A (TSA) or DMSO. After 24-h transfection, the cells were lysed, and the luciferase activity was measured by a Berthold LB9507 luminometer. The luciferase activity was normalized by *Renilla*. The level of activation was expressed relative to the luciferase activity obtained after cotransfection of the pCMV alone. The histogram represents the average of three independent sets of transfection experiments with the error bars indicating one s.d.

Figure 5. Effects of CRIF1 and its mutants on the Nur77 transactivation function. (**A**) Schematic representation of the CRIF1 wild type and deletion mutants. (**B**) The C2C12 cells were transiently cotransfected with the expression plasmids encoding 0.1μg CRIF1 and the 0.1μg CRIF1 mutants with 0.1μg Gal4-Nur77-AB, along with the reporter plasmid 0.2μg Gal4-tk-Luc. After 24-h transfection, the cells were lysed, and the luciferase activity was measured by a Berthold LB9507 luminometer. The luciferase activity was normalized by *Renilla*. The activation level was expressed relative to the luciferase activity obtained after cotransfection of the pCMV alone. The histogram represents the average of three independent sets of transfection experiments with error bars indicating one s.d.

Figure 6. Knockdown of the CRIF1 protein increases transactivation of Nur77. (A) C2C12 skeletal muscle cells were cultured in 12 well plates until 80% confluence and cotransfected with 0.2μg Gal4-Tk-Luc reporter construct and with or without **(A)** 0.2μg Gal4-Tk-Luc reporter construct and with or without **(A)** 0.1μg of Gal4- Nur77, **(B)** 0.1μg of Gal4-Nur77-AB, 0.5μg of CRIF1-siRNA duplexes or 0.1μg of pFLAG-CRIF1 added, After a 24-h transfection, cells were lysed, and luciferase activity was measured by a Berthold LB9507 luminometer. Luciferase activity was normalized by *Renilla.* Fold activation was expressed relative to luciferase activity obtained after cotransfection of the pCMV alone. The histogram represents the average of three independent sets of transfection experiments with error bars indicating one s.d. upper panel of **(A)** shows the Western blot analysis CRIF1 expression in cells. the C2C12 cells were cultured in six-well plates until 80% confluence and transfected with the CRIF1-siRNA duplexes (500 ng/well) using LipofectAMINE Plus (Invitrogen). After a 24-h transfection, the total lysates were prepared and blotted with anti-CRIF1 and anti-β-actin antibodies.

Figure 7. Induction and Activation of Nur77 by TSH. (A) The FRTL-5 thyroid cells were grown to near confluence in Coon's modified Ham's F-12 medium containing 5% (v/v) calf serum. The cells were maintained for 6 days with 5H medium not containing TSH and serum. The medium was replaced with fresh medium including the following additions as indicated: the FRTL-5 thyroid cells were maintained in 4H without serum condition for 48hr and treated with TSH

(1mU/ml), NurRE confer a higher responsiveness to the TSH treatment. The cells were treated with TSH or without TSH (4H5). The FRTL-5 thyroid cells were cotransfected with 0.2μg of NurRE, 0.01-0.2μg of CRIF1 and assayed for their luciferase activity after 48h. **(B)** The C2C12 skeletal muscle cell cultured 12 well plate and cotransfected with 0.2μg of the NurRE reporter construct and with or without **(B)** 0.1μg of the Nur77, and cotransfected with 0.2μg of the Gal4-Tk-Luc reporter construct and with or without **(C)** 0.1μg of the Gal4-Nur77-AB, 0.01-0.2μg of the CRIF1 with 0.1μg of the PKA-catalytic domain. After 24-h transfection, the cells were lysed, and the luciferase activity was measured by a Berthold LB9507 luminometer. The luciferase activity was normalized by *Renilla*. The level of activation was expressed relative to the luciferase activity obtained after cotransfection of the pCMV alone. The histogram represents the average of three independent sets of transfection experiments with the error bars indicating one s.d.

LexA	B42	Interaction
-	-	-
CRIF1	-	-
-	CRIF1	-
ERRγ	CRIF1	-
GR	CRIF1	-
SHP	CRIF1	-
mAR	CRIF1	+
SF-1	CRIF1	-
CRIF1	RXR	-
CRIF1	CAR	-
CRIF1	TR	-
CRIF1	SMRT	-
CRIF1	Nur77	++++

Table 1. Characterization of the novel CRIF1 and Nur77 interaction. (A) The plasmids encoding the Lex A- DBD fusion of CRIF1, ERRγ, GR, SHP, mAR and SF-1 with the plasmids encoding the B42-AD fusion of CRIF1, RXR, CAR, TR, SMRT and Nur77 were transformed into the yeast strain, EGY48. The amount of β-gal activity was measured as described in Materials and Method. The positive interactions are indicated as **+** and negative as **-**.

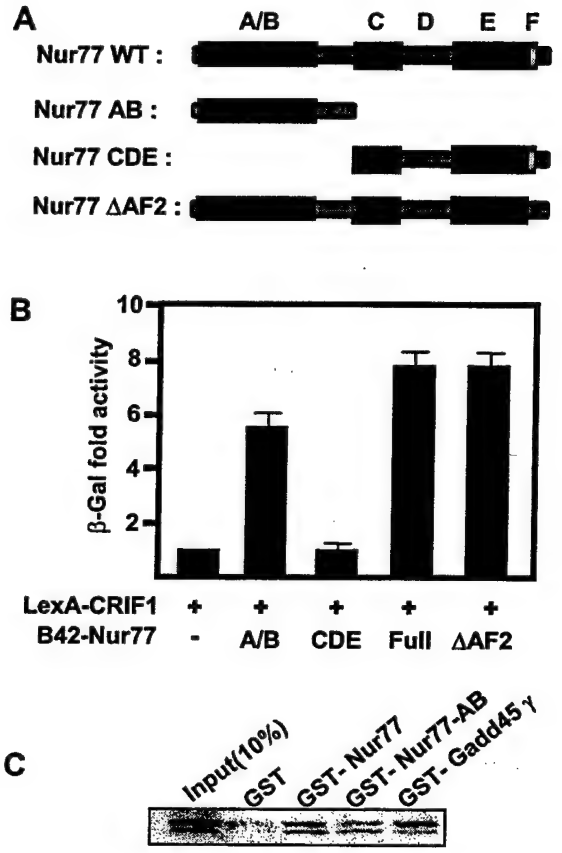
A
A/B
C
D
E
F
Nur77 WT :
Nur77 AB :
Nur77 CDE :
Nur77 ΔAF2 :
B
β-Gal fold activity
10
8
6
4
2
LexA-CRIF1 + + + + +
B42-Nur77 - A/B CDE Full ΔAF2
C
Input(10%)
GST
GST- Nur77
GST- Nur77-AB
GST- Gadd45γ

Fig 1.

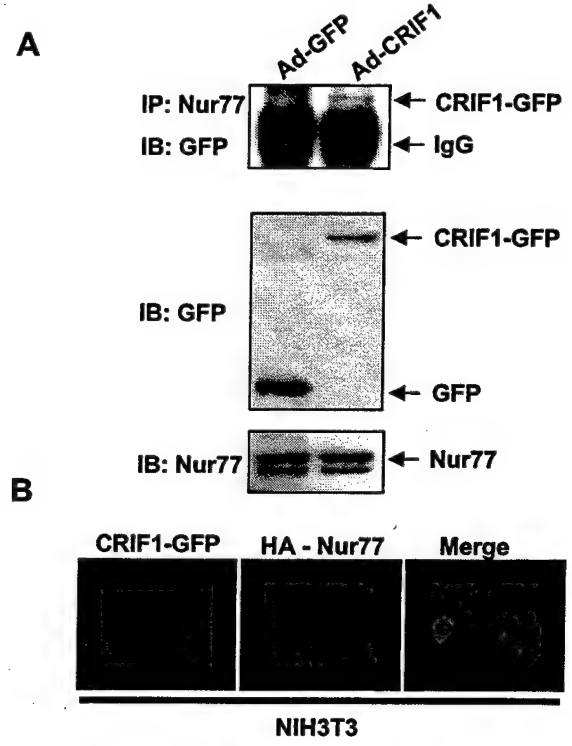
A
Ad-GFP
Ad-CRIF1
IP: Nur77
IB: GFP
CRIF1-GFP
IgG
IB: GFP
CRIF1-GFP
GFP
IB: Nur77
Nur77
B
CRIF1-GFP
HA - Nur77
Merge
NIH3T3

Fig 2.

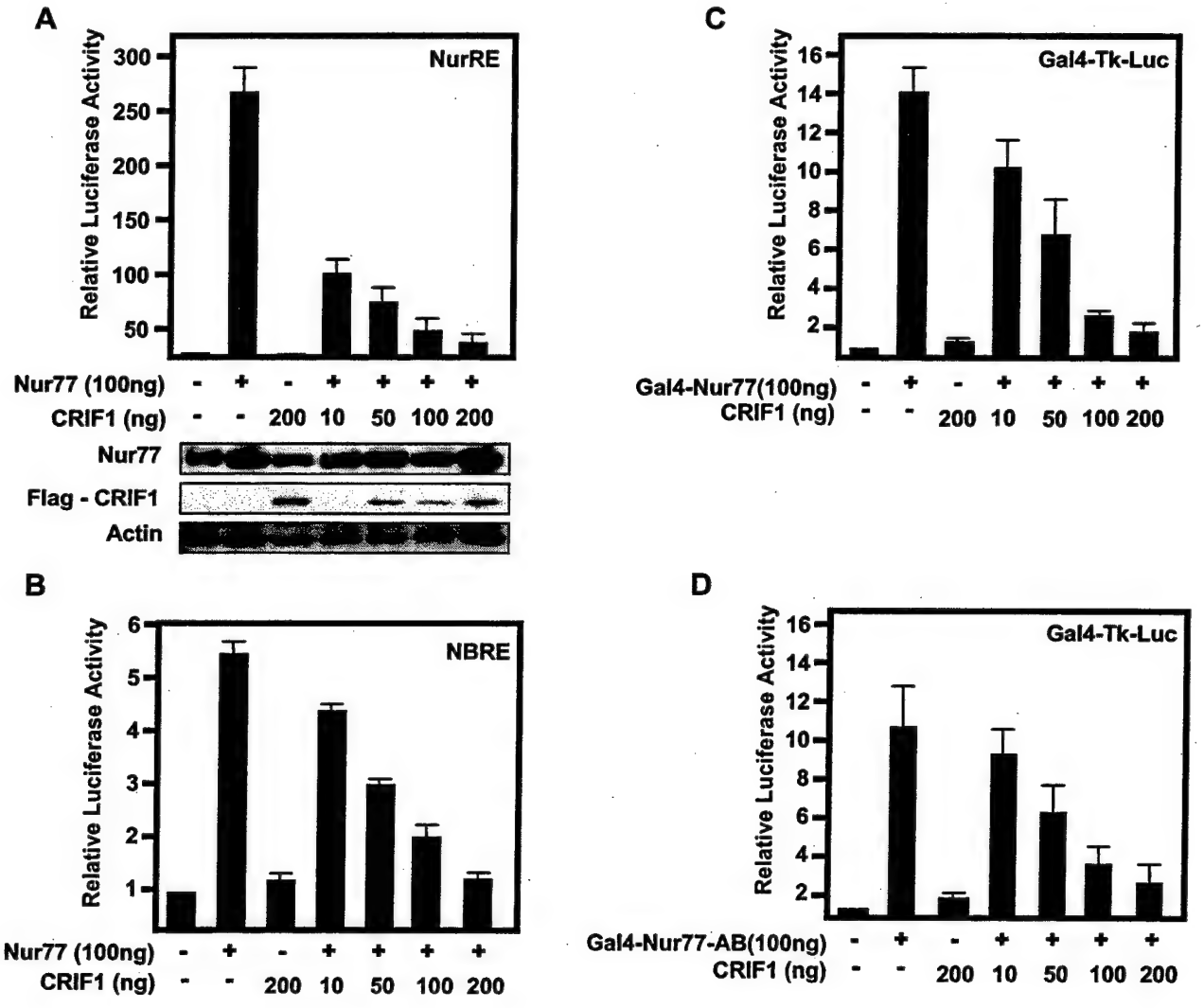
A
NurRE
Relative Luciferase Activity
300
250
200
150
100
50
Nur77 (100ng) - + - + + + +
CRIF1 (ng) - - 200 10 50 100 200
Nur77
Flag - CRIF1
Actin
B
NBRE
Relative Luciferase Activity
6
5
4
3
2
1
Nur77 (100ng) - + - + + + +
CRIF1 (ng) - - 200 10 50 100 200
C
Gal4-Tk-Luc
Relative Luciferase Activity
16
14
12
10
8
6
4
2
Gal4-Nur77(100ng) - + - + + + +
CRIF1 (ng) - - 200 10 50 100 200
D
Gal4-Tk-Luc
Relative Luciferase Activity
16
14
12
10
8
6
4
2
Gal4-Nur77-AB(100ng) - + - + + + +
CRIF1 (ng) - - 200 10 50 100 200

Fig 3.

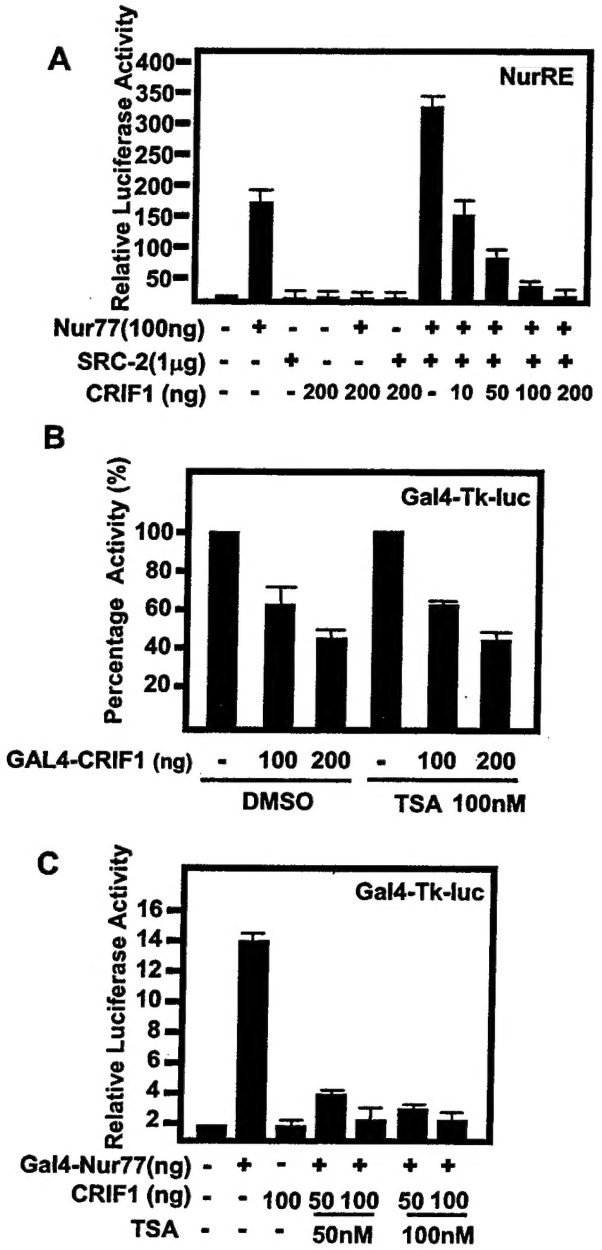
A
Relative Luciferase Activity
400
350
300
250
200
150
100
50
NurRE
Nur77(100ng) - + - - + - + + + + +
SRC-2(1μg) - - + - - + + + + + +
CRIF1 (ng) - - - 200 200 200 - 10 50 100 200
B
Percentage Activity (%)
100
80
60
40
20
Gal4-Tk-luc
GAL4-CRIF1 (ng) - 100 200 - 100 200
DMSO
TSA 100nM
C
Relative Luciferase Activity
16
14
12
10
8
6
4
2
Gal4-Tk-luc
Gal4-Nur77(ng) - + - + + + +
CRIF1 (ng) - - 100 50 100 50 100
TSA - - - 50nM 100nM

Fig 4.

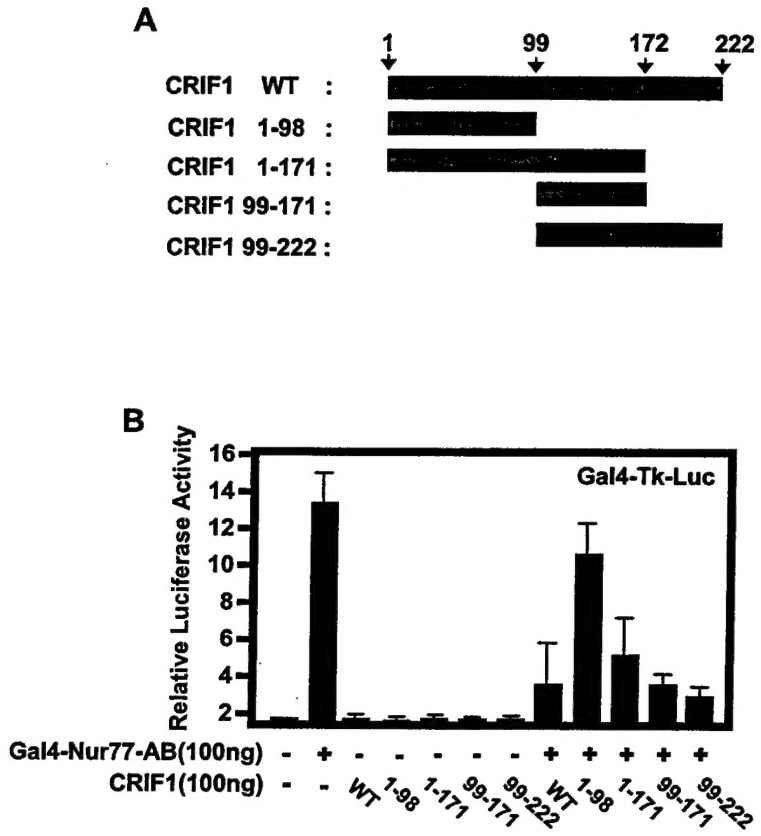
A
1
99
172
222
CRIF1 WT :
CRIF1 1-98 :
CRIF1 1-171 :
CRIF1 99-171 :
CRIF1 99-222 :
B
Relative Luciferase Activity
16
14
12
10
8
6
4
2
Gal4-Tk-Luc
Gal4-Nur77-AB(100ng) - + - - - - - + + + + +
CRIF1(100ng) - - WT 1-98 1-171 99-171 99-222 WT 1-98 1-171 99-171 99-222

Fig 5.

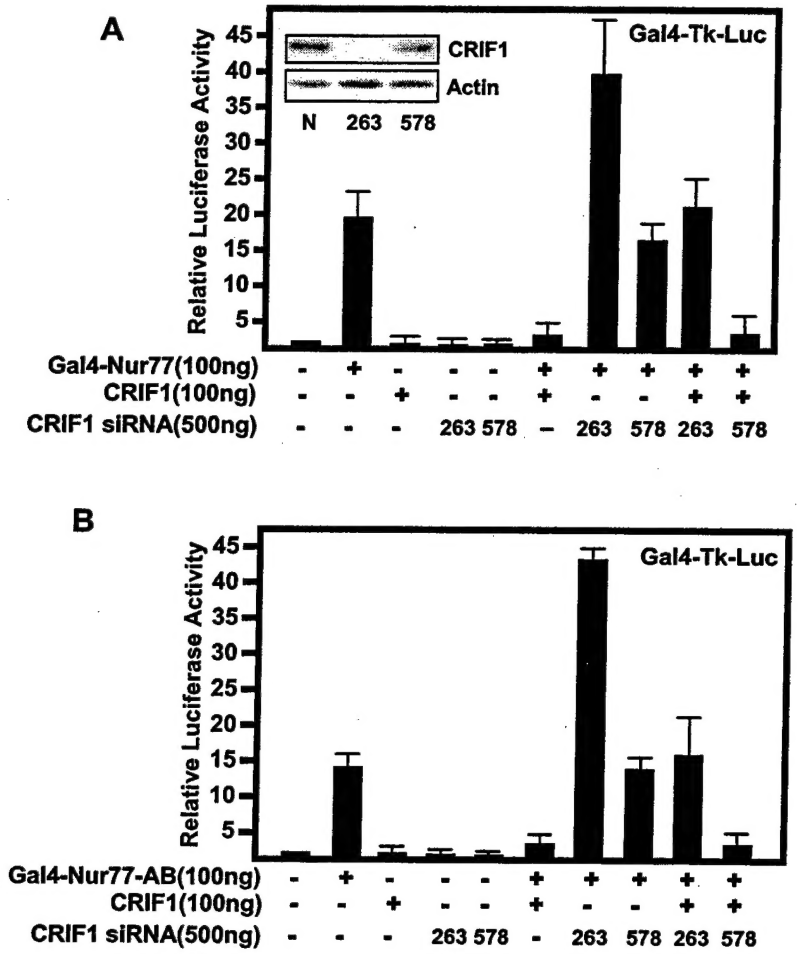
A
Relative Luciferase Activity
45
40
35
30
25
20
15
10
5
CRIF1
Actin
N 263 578
Gal4-Tk-Luc
Gal4-Nur77(100ng) - + - - - + + + + +
CRIF1(100ng) - - + - - + - - + +
CRIF1 siRNA(500ng) - - - 263 578 – 263 578 263 578
B
Relative Luciferase Activity
45
40
35
30
25
20
15
10
5
Gal4-Tk-Luc
Gal4-Nur77-AB(100ng) - + - - - + + + + +
CRIF1(100ng) - - + - - + - - + +
CRIF1 siRNA(500ng) - - - 263 578 - 263 578 263 578

Fig 6.

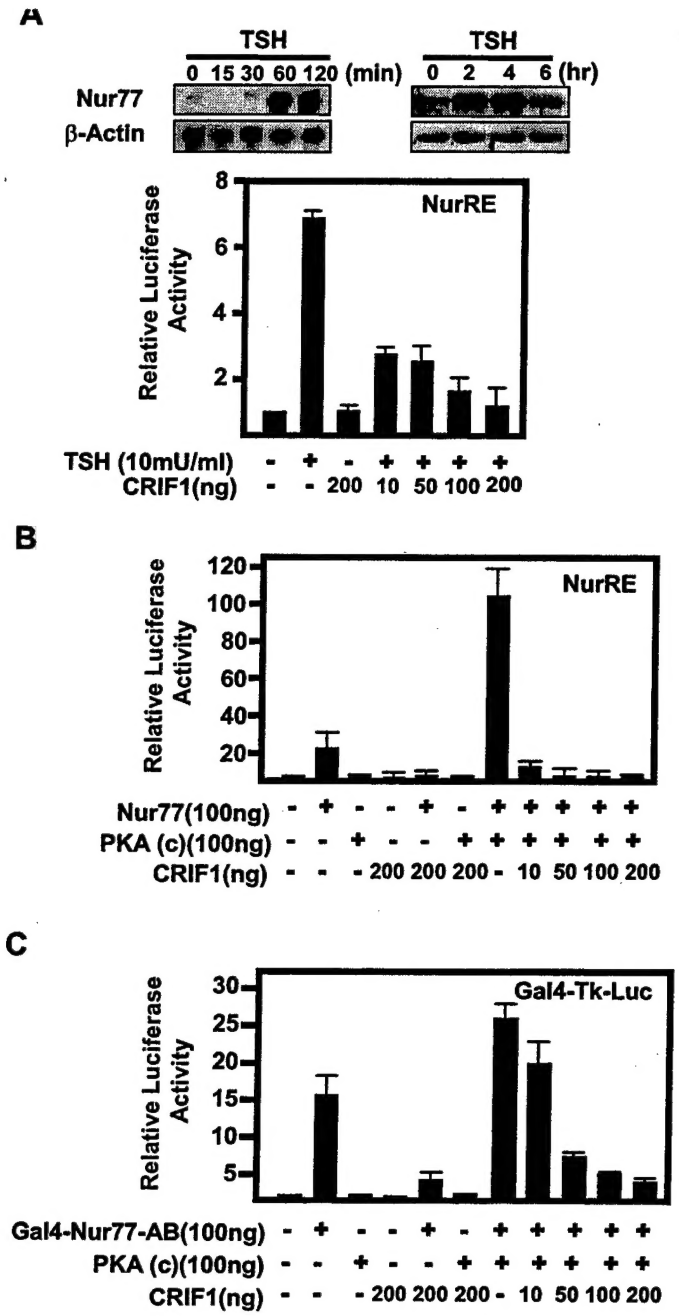
A
TSH
0 15 30 60 120 (min)
TSH
0 2 4 6 (hr)
Nur77
β-Actin
Relative Luciferase Activity
NurRE
TSH (10mU/ml)
CRIF1(ng)
B
Relative Luciferase Activity
NurRE
Nur77(100ng)
PKA (c)(100ng)
CRIF1(ng)
C
Relative Luciferase Activity
Gal4-Tk-Luc
Gal4-Nur77-AB(100ng)
PKA (c)(100ng)
CRIF1(ng)

Fig 7.